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The Role of Monocyte Chemoattractant Protein-1 on Operant Ethanol Self-Administration in Long-Evans Rats

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The Role of Monocyte Chemoattractant Protein-1 on Operant Ethanol Self-Administration in Long-Evans Rats

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Dedication

I dedicate this dissertation to my parents Pavel and Irena Valenta, the hardest-working people I have ever met.

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The Role of Monocyte Chemoattractant Protein-1 on Operant Ethanol Self-Administration in Long-Evans Rats

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The neuroimmune system can exert a powerful influence over behavior, and evidence is mounting that the neuroimmune system can influence the intake of drugs of abuse. Insight into the interaction between drugs of abuse and the neuroimmune system will teach us more about the inner workings of the brain and can lead to new treatment options for addiction. Previous research has demonstrated that alcoholics have elevated levels of immune signaling molecules. My dissertation project demonstrated that elevating immune signaling molecules in the brain can directly increase ethanol consumption.

A variety of cytokines are elevated in human alcoholics and animal models of ethanol dependence. However, recent research has suggested that monocyte chemoattractant protein -1 (MCP-1) is particularly important. Researchers have found elevated levels of MCP-1 in the brains of human alcoholics, in animal brains after chronic exposure to ethanol, and in brain slices exposed to ethanol. Also, MCP-1 or MCP-1 receptor (CCR2) knockout mice had a significant reduction in ethanol consumption and a reduced preference for ethanol. My goal was to clarify if MCP-1 signaling could increase ethanol intake.

Generally speaking, I accomplished this by increasing the amount of MCP-1 signaling in the brain and then measuring ethanol drinking behavior. I infused MCP-1 into the cerebral ventricles of rodents for 4 weeks and measured their ethanol intake for those 4 weeks as well as 4 additional weeks. There was a significant interaction between dose of MCP-1 and ethanol consumed across the first 4 weeks (while pumps were flowing) and across the 8-week experiment. Animals receiving the highest dose of MCP-1 (2 μg/day) were the highest consumers of ethanol during weeks 3 through 8.

My second goal was to determine how the modulation of brain MCP-1 signaling could influence drinking behavior in ethanol-dependent rodents. I made progress toward this goal by reliably reaching target BAC's in rodents through the use of ethanol vapor inhalation chambers, but I did not reach the point of inducing dependence or modulating MCP-1.

The neuroimmune system seems to be paramount in the progressive loss of control over drug intake seen in drug addiction and presents a potential route for the treatment of addiction. The results of my experiments support this hypothesis by providing evidence that neuroimmune signaling can increase ethanol consumption, show that MCP-1 signaling is critical in this phenomenon, and identify MCP-1 signaling as a strong candidate for investigating the therapeutic potential of neuroimmune signaling for alcohol use disorders.

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Chapter 1: General Introduction

Alcohol abuse and alcohol dependence are among the most widespread and costly health concerns in the United States. An estimated 17.6 million American adults (8.5 percent) are afflicted with one of the two disorders every year (Hasin et al., 2007) with an estimated economic cost of $223.5 billion per year (Bouchery et al., 2011). Notably, 30.3 percent of Americans have been afflicted with it at some point in their life (Hasin et al., 2007). Like other substance abuse disorders, it can be seen as a progressive loss of behavioral control that leads to continued use despite harm. The neurobiological mechanisms that contribute to the shift from healthy to unhealthy alcohol consumption are complex and mostly undiscovered. With only a few unreliable treatment options available, the development of new molecular targets is critical.

Alcohol use disorders (AUD's) have profound effects on the immune system, including the immune system of the brain. There is evidence of an interaction between AUD's and neuroimmune signaling or neuroinflammation, including the following: alcoholic human brains have increased amounts of neuroimmune signaling molecules (cytokines), increasing neuroimmune signaling leads to increased ethanol consumption in animals, and genetically modified mice lacking specific components of their immune system tend to drink less ethanol than their counterparts.

A variety of cytokines are elevated in human alcoholics and animal models of dependence. However, recent research has suggested that monocyte chemoattractant protein -1 (MCP-1) is particularly important. MCP-1 is a central molecule in the inflammatory response and recruits microglia to sites of brain injury (Piao et al., 2008). Researchers have found elevated levels of MCP-1 in the brains of human alcoholics, in animal brains after chronic exposure to ethanol, and in brain slices exposed to ethanol. MCP-1 receptor (CCR2) knockout mice had a robust reduction in ethanol consumption and a reduced preference for ethanol (Blednov et al., 2005). Altogether, this data suggests that: MCP-1 signaling is important in the development of drinking behavior of wild-type rodents; that enhancing MCP-1 signaling may accelerate the onset of ethanol dependence or increase the severity of ethanol dependence; that blocking MCP-1 signaling may blunt the development of drinking behavior; and identifies MCP-1 and it's receptor as potential target molecules for therapeutic intervention. The goal of my dissertation is to test the hypothesis that MCP-1-mediated neuroimmune signaling can influence ethanol self-administration.

Generally speaking, I accomplished this by increasing the amount of MCP-1 signaling in the brain and then measuring drinking behavior. There are three phases of drinking behavior I investigated. First, I looked at the acquisition of drinking behavior (i.e. an animal that has never drunk before was given a chronic infusion of MCP-1 and I measured how fast the animal began to drink and how fast the drinking escalated). After a week or so, the animals generally stabilized their ethanol intake, and I measured if the

MCP-1 infusion influenced the average daily intake during "stable" intake for several weeks. Third, I looked at how long the influence on drinking lasted after the MCP-1 infusion was stopped.

In short, there was a significant interaction between dose of MCP-1 and sweetened ethanol consumed across the first 4 weeks (while pumps were flowing) and across the 8-week experiment. Animals receiving the highest dose of MCP-1 (2 µg/day) were the highest consumers of ethanol during weeks 3 through 8. MCP-1 did not influence the acquisition of self-administration (measured across the first 5 days), the motivation to consume ethanol (time to lever press or progressive ratio), withdrawal-induced anxiety, or the consumption of sucrose alone.

The mechanisms influencing ethanol intake during casual drinking compared to drinking while dependent on ethanol (i.e. alcoholism) are different, and the influence of neuroimmune signaling on each is potentially distinct. With a variety of research suggesting that neuroimmune signaling interacts with ethanol dependence, I also attempted to research how the modulation of brain MCP-1 signaling could influence drinking behavior in dependent rodents. In order to induce dependence, I built an ethanol vapor inhalation chamber system. Vapor inhalation has been shown to increase ethanol drinking in various strains of rodents. I have also replicated a common home cage drinking model with the goal of using ethanol vapor to influence home cage ethanol intake. However, the rate of alcohol metabolism between individual Long-Evans rats seemed highly variable,

and I had some trouble obtaining reliable blood alcohol levels in my rodents using ethanol vapor. However, reliable data was obtained when using 4-methylpyrazole to block ethanol metabolism. I have not yet tested the influence of MCP-1 on drinking behaviors of dependent animals. However, both the home cage drinking data and the vapor inhalation data will be presented in this dissertation.

My research has provided novel evidence that neuroimmune signaling can directly increase chronic operant ethanol self-administration, and that this increase persists beyond the administration of the cytokine. These data suggest that ethanol-induced increases in MCP-1, or increases in MCP-1 due to various other neuroimmune mechanisms, may further promote ethanol consumption. By understanding how neuroimmune signaling can regulate drinking behavior, we can develop insights into the transition from healthy to unhealthy drinking behavior and we can develop new molecular targets for treatment. Continued research into the MCP-1 mechanism, particularly using models of ethanol dependence, will help determine if targeting MCP-1 signaling has therapeutic potential in the treatment of alcohol use disorders.

THE BRAIN'S INNATE IMMUNE SYSTEM

Nervous tissue contains 4 major classes of cells: neurons, astrocytes, oligodendrocytes, and microglia. Neurons are highly specialized cells that are electrically excitable and form a complex network of neurotransmitter-based intercellular

communication that is the foundation of thoughts and consciousness. Astrocytes, oligodendrocytes, and microglia are collectively referred to as glia or glial cells and are generally considered supporting cells for neurons but have specialized responsibilities as well. Oligodendrocytes wrap the axons of neurons in myelin sheaths, helping them propagate electrical signals efficiently. Astrocytes provide mechanical and biochemical support to neurons and other cells, including endothelial cells in the brain. They participate in tissue repair mechanisms, ion transport, neurotransmitter uptake and release, and help regulate ion concentrations. Microglia are macrophages that form the basis of the brain's immune system. They are mobile within the brain and communicate with other cells primarily through the secretion of cytokines.

Cytokines are molecules that immune cells use for cell-to-cell communication. Chemokines are a type of cytokine that induces chemotaxis of cells up the concentration gradient. For example, MCP-1 is a chemokine that attracts monocytes. There are dozens of chemokines, but only 19 known receptors, all of which are metabotropic, g-protein coupled receptors.

The blood-brain barrier (BBB) is a selectively permeable barrier formed by endothelial tight-junctions that aids in maintaining a highly specific environment for central nervous tissue and helps prevent the spread of infections to the delicate nervous tissue. The BBB is tightly regulated and prevents all but the smallest molecules from passively diffusing into the brain parenchyma. Astrocytes generally wrap around the

junctions between endothelial cells, provide biochemical support for the endothelial cells and participate in the transport of molecules across the barrier, but evidence shows a limited involvement in the barrier itself (Kimelberg et al., 1993). Regulation of the barrier is a complex process which can be dramatically influenced by the neuroimmune system, particularly through microglial cytokine secretions.

Innate immunity is known as the "first line of defense" against pathogens and constitutes a fast, nonspecific response to pathogen invasion. Innate mechanisms include inflammation, which functions to increase the flow of immune cells to the area as well as providing a physical barrier to the spread of pathogens. This is in contrast to the adaptive immune system, which provides a slow, albeit specific response to a pathogen that is "remembered" by pathogen-specific t-cells which can mount a fast and powerful response in the future. The brain was originally considered an "immunoprivileged" site due to the lack of adaptive immune mechanisms. It was thought the only interaction between the neuroimmune system and the peripheral immune system occurred during the traditional neuroinflammatory response due to tissue damage or neurodegenerative disease, defined by leukocyte infiltration across the BBB and microglial activation. Antibodies produced by the adaptive immune system, and most cytokines, are too large to passively diffuse through the BBB. However, recent research has demonstrated an active and tightly regulated immune system of the CNS that communicates with the peripheral immune system through a variety of other mechanisms.

Neuroimmune signaling certainly has a traditional role in the neuroinflammatory response to pathogens, tissue damage, or a variety of other pathological states. However, recent research has shown that the interaction between the nervous system and the neuroimmune system is far more complex than previously imagined. For example, recent evidence has even illuminated the importance of the neuroimmune system in "normal" mechanisms, such as neuronal function, signal transduction, plasticity, neuroprotection, and neurodevelopment (for a review, see Réaux-Le Goazigo et al., 2013). Furthermore, neuroimmune mechanisms have been shown to influence complex behaviors such as "sickness behavior" and alcohol drinking patterns, likely through interactions with neurons. Even the presence of elevated levels of microglia or cytokines does not necessarily indicate a neuroinflammatory state because of their role in normal brain functions.

MICROGLIA

Microglia are the resident macrophages of the brain and spinal cord (Lawson et al., 1990) and account for about 15% of the cells found in the brain (Perry, 1998). Microglia are the foundation of the brain's (innate) immune system and are the only mononuclear phagocytic cell in the brain under normal conditions. Therefore, they provide the first line of defense to brain insults and are directly responsible for detecting pathogens and tissue damage (e.g. trauma, *Streptococcus pneumoniae*, etc). Naturally, they are paramount to the initiation of an immune response and can recruit help from peripheral immune cells

through the BBB by regulating chemotaxis and endothelial tight junctions (Stamatovic et al., 2005; Cushing and Fogelman, 1991; Tieu et al., 2009, Gunn et al., 1997).

The origin of microglia has been debated for decades. Although it's possible for blood-derived monocytes to cross through the BBB and differentiate into microglia under specific circumstances (Lawson et al., 1992), emerging evidence is suggesting that embryonic precursors proliferate in the CNS prior to blood hematopoiesis (Ginhoux et al., 2013). Additional microglia are observed during inflammation (reactive microgliosis) and the source of these is under debate, with a high likelihood of both local proliferation and infiltration from the blood (Ginhoux et al., 2013; Capotondo et al., 2012). In either case, they are the only mesoderm-derived cells in the brain.

Microglia undergo stages of activation due to the initiation of activating signals or a loss of constitutive inhibitory signaling (such as CX3CR1). Activators of microglia include toxins, dead cells, virus and bacteria constituents, damaged neurons, blood serum constituents (BBB leakage), neurotransmitters, and activated astrocytes. Local cytokines can prime or activate microglia, but it is also important to mention that various cytokines from the periphery can also prime or activate microglia, including TNFα or MCP-1 (Ferrari and Tarelli, 2011). "Resting" microglia exhibit a ramified morphology which reflects their constant scanning of their environment for danger signals. Activated microglia take on an "amoeboid" or "bushy" shape and both migrate and proliferate in response to endogenous or exogenous signals (Monk and Shaw, 2006). Activation of microglia can be fast and

long-lasting, and activated microglia increase their expression of characteristic genes. However, activated microglia can have several different phenotypes during activation, including various neurotrophic or neurotoxic phenotypes (Colton, 2009). Raivich et al. (1999) consider 4 states of microglia activation, each with characteristic morphology and cytokine secretions. "Stage 1" morphology is "alert" or "primed" with thicker but still ramified processes and secretions of TGF-B1. "Stage 2" activation includes migratory/proliferating bushy microglia that secrete IL-10. Both stage 1 and stage 2 are mostly neurotrophic and can increase clearance of toxic materials. Stage 3a and 3b exhibit amoeboid morphology and each has characteristic inflammatory cytokine secretions. Stage 3a microglia are phagocytic while 3b are mostly bystanders (Raivich et al., 1999). Microglia can shift between stages based on environmental stimuli (Kettenmann et al., 2011). In chronic neuroinflammation, microglia can stay active for extended periods. Experienced microglia could return to a resting state or stay in a primed state, and primed microglia "could reveal an altered responsiveness and exert distinct responses upon re-challenge" (Kettenmann et al., 2011).

Astrocytes also participate in the neuroinflammatory response, including the secretion of cytokines, and can, therefore, influence amplification cascades (Block et al., 2007). The extent of astrocytic involvement is heavily dependent on microglial factors, but is not completely understood (Holm et al, 2012). Astrocytic responses include increasing neuronal excitability (Pascual et al., 2012) and the release of glutamate (Jourdain et al., 2007), which could influence neuronal function and/or dysfunction and

activate neuroimmune responses. Microglia can also use cytokine signaling to influence astrocytic involvement, including IL-4 to suppress astrocyte function and IL-1 to increase astrocyte function (Gehrman et al., 1995). However, astrocytes seem to be most active during the late, recovery stages of inflammatory diseases, rather than initiating inflammation (Gehrman et al., 1995).

Microglia can also interact with neurons during both normal and inflammatory processes. Both secrete cytokines, as well as neurotransmitters and both, have receptors for cytokines and neurotransmitters (Blalock, 1989). Neurons are capable of controlling the extent of microglial reactivity through direct cell-to-cell interactions, including neurotransmitter release as well as cytokine signaling (Carnevale et al., 2007). This includes microglial receptors for noradrenaline, which increases microglial proinflammatory cytokine signaling, as well as acetylcholine, which attenuates proinflammatory cytokine signaling (Carnevale et al., 2007). Neurons can also activate microglia when damaged, particularly when cell contents are released (e.g. HMGB1). HMGB is a common nuclear protein and a powerful endogenous activator of TLR4 receptors. HMGB is released when cell membrane integrity is lost, and there is some evidence that it can be secreted through NF-kB activation (for a review, see Lotze and Tracey, 2005). Neurons can also produce TNFα and IL-1, which can be used to communicate with other neurons as well as microglia (Breder et al., 1988; Breder et al., 1994).

Microglia can also communicate with neurons through the release of neurotransmitters as well as neuropeptides such as endorphins (for a review, see Domercq et al., 2013). Microglia can also secrete neurotrophic or neurotoxic cytokines depending on the context. Neurotoxic events include the release of prostaglandins, reactive oxygen species, proteases, or the removal of neurotrophic factors. Research is proving that microglial neurotoxicity is central to the progression of neurodegenerative disorders. Microglial activation or microglial-induced neuroinflammatory processes occur in "virtually all brain pathologies" (Ginhoux, 2013) through direct interaction with neurons (Perry et al., 2010; Kettenmann et al., 2011; Kingwell, 2012). For example, microglial expression of IL-1 or MCP-1 is associated with neuronal damage in Alzheimer's disease (Mrak and Griffin, 2005; Ishizuka et al., 1997). Microglial induced neurotoxicity is also implicated in Parkinson's Disease, multiple sclerosis, and most other neurodegenerative diseases. Microglia have also been known to influence neuronal excitability in the CNS or PNS, and this excitability has been linked to neuropathic pain (Watkins and Maier, 2002).

Although many cytokines cannot cross the BBB, the CNS is still capable of communicating with the peripheral immune system and vice versa. Peripheral inflammation has been known to influence neurotransmitter release in the brain, including increasing neuronal firing by 100% in the hypothalamus (Besedovsky et al., 1977). Furthermore, brain administration of cytokines or lipopolysaccharide can also activate the HPA axis, which then results in the release of glucocorticoids, which can suppress cytokine

signaling in the periphery or travel back to the brain and suppress cytokine signaling there (Wexler, 1957).

It is important to stress that although communication between microglia and neurons or other cells occurs under pathological conditions, microglia also have important functions under "normal" conditions as well. Many of the aforementioned communication pathways occur under normal non-inflammatory conditions, although generally to a lesser extent. Evidence shows that microglial functions are critical for neuronal proliferation, differentiation, and synaptic formation, pruning, and remodeling (Hughes, 2012; Graeber, 2010) and respond to neurotransmitters such as glutamate, dopamine, GABA, and purines (Graeber, 2010), with acetylcholine having well-known anti-inflammatory properties (Borovikova et al., 2000).

NEUROIMMUNE SIGNALING, STRESS, AND BEHAVIOR

The relationship between stress and drug abuse is strong and particularly prevalent in the literature. Evidence is suggesting that neuroimmune signaling may be a mechanism through which this relationship is mediated.

To begin, the relationship between neuroimmune signaling and behavior in general is very complex and interrelated. For example, sickness or infection is known to have a powerful influence on brain functions and complex behaviors (for a review, see Dantzer et

al., 2008). The presence of cytokine receptors on neurons is one mechanism through which sickness-like behaviors are expressed (Tracey, 2009). For example, the interaction between the endogenous danger-signaling molecule HMGB-1 or exogenous lipopolysaccharide with the TLR4 receptor can influence neurotransmission and result in sickness-like behaviors (Blednov et al., 2011; Okun et al., 2009; Lehnardt, 2010; Andersson and Tracey, 2011). Other cytokines including TNFα and IL-6 are known to promote sickness behavior in similar ways. Another example of neuroimmune systems affecting behavior is that intracerebroventricular (ICV) administration of IL-1 is known to impair contextual memory in fear conditioning behavior (Barrientos, 2002).

Furthermore, stress and depression are associated with increased levels of cytokines in humans and animal models. This appears to be primarily through cytokine-induced dysregulation of the HPA axis as well as dopamine and serotonin systems (Dantzer et al., 2008). As previously mentioned, cytokines are known to increase the release of corticotropin release hormone (CRH) or cortisol (Hueston & Deak, 2014), which are colloquially referred to as stress hormones and can have a dramatic impact on many neurotransmitter systems, especially those involved in the regulation of mood. The resulting increase in glucocorticoids can also have a dramatic impact on cytokine signaling in the brain or periphery (Munhoz et al., 2006).

The association between stress systems and the neurobiology of addiction is particularly strong, especially in the neurobiology of alcohol use disorders. CRH systems

are central in driving addictive-like behaviors through actions in the central extended amygdala (CeA), where they act to produce anxiety, anhedonia, compulsive intake of drugs, and reinstatement of extinguished drug seeking (For a review, see Zorrila et al., 2014). Since stress, mood, and immune function are closely related, particularly in their interaction with the HPA axis, neuroimmune mechanisms may be integral in the link between stress and drug abuse.

NEUROIMMUNE SIGNALING AND DRUG ABUSE

Evidence is mounting that the neuroimmune system participates in the progressive pathological deterioration into drug addiction (for a review, see Crews et al., 2011). The influence of neuroimmune signaling on neurotransmitter activity, HPA axis/endocrine function, and CNS development are just a few examples of this link. Current drug abuse models typically distinguish between various stages, including binge/intoxication, withdrawal/negative affect, and preoccupation/anticipation or craving (for a review, see Mayfield et al., 2013). Neuroimmune systems (as well as stress systems), have been shown to influence all three stages of addiction. For example, blockade of microglial activation prevents conditioned place preference for opioids, stimulants, or alcohol (Narita et al., 2006; Hutchinson et al., 2008; Agrawal et al., 2011) and reduces, by half, the dopamine increases seen by morphine administration (Bland et al., 2009), strong evidence that neuroimmune function is involved in the binge/intoxication stage of drug abuse.

The negative affect, anhedonia, and reward-deficits seen as a result of the administration of innate immune cytokines (or with stress or depression) are akin to the behavioral changes seen in drug abuse disorders and are associated with the dysregulation of limbic function (Figure 1.1). Drugs of abuse or restraint stress are known to increase the release of cytokines and activate microglia (Blanco and Guerri, 2007; Crews et al., 2012; Madrigal et al., 2002; Tynan et al., 2010), which, as previously mentioned, contribute to dysregulation of the HPA axis as well as dysregulation of dopamine and serotonin systems (Dantzer et al., 2008). Also, glial activation and cytokine release is associated with increased excitability of reward pathways (Ren et al., 2012). Furthermore, mice lacking TLR4 receptors are protected against microglial activation, anxiety and cognitive impairments seen with chronic alcohol administration (Pascual et al., 2011), drink less ethanol than normal mice (Blednov et al., 2012), and have decreased activation of the amygdala (Roberto et al., 2008). LPS administration is also associated with decreased firing of dopamine neurons (Blednov et al., 2011). Furthermore, brain infusions of cytokines (including MCP-1) are associated with increased withdrawal-induced anxiety (Breese et al., 2011).

Drugs of abuse to are known to cause cognitive impairment, particularly in prefrontal function (Schoenbaum and Shaham, 2008), and "neuronal excitability," both of which are associated with increased craving and compulsive drug use. Neuroimmune signaling is associated with both cognitive impairments as well as neuronal excitability.

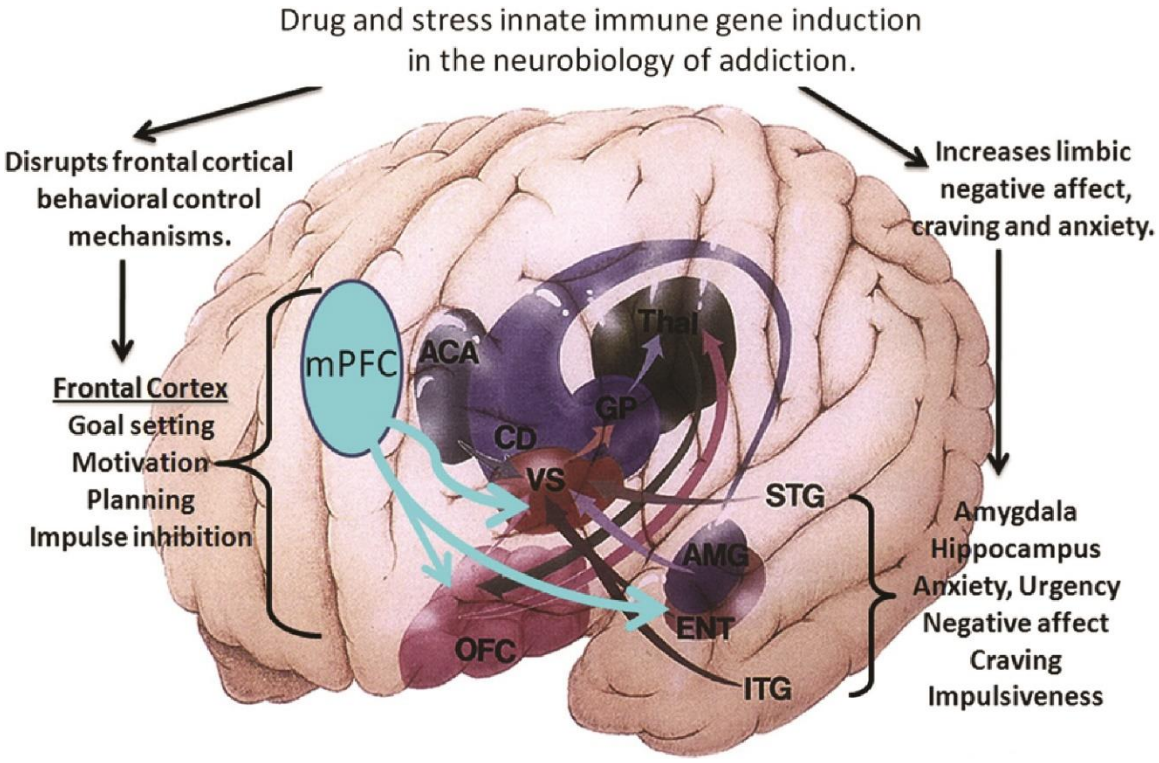

Figure 1.1: Innate immune gene induction creates the neurobiology of addiction.

Prefrontal control over decision making as well as control over limbic structures is compromised due to a hyperglutamatergic state. Neuroimmune and stress systems interact to induce negative affect, craving and anxiety through limbic dysfunction. Adapted from Crews et al. (2011) with permission.

For example, neuroimmune activation is associated with the reduction of seizure thresholds in the corticolimbic system (Vezzani et al., 2011; Maroso et al., 2010). Furthermore, the release of glutamate is associated with microglial activation and IL-1 release, and neuroimmune signaling increases the phosphorylation of synaptic glutamate receptors (Maroso et al., 2010). The cytokine TNFα increases glutamate neurotoxicity by activating NMDA receptors (Zou and Crews, 2005). Hyperglutamatergic states, which can be cytokine-induced, are known to disrupt prefrontal control over limbic systems (Figure 1.1; Gruber et al., 2010). These hyperexcitable states can be associated with craving for drugs of abuse (Breese et al., 2011). A hyperglutamatergic state has been found to increase alcohol intake (Spanagel et al., 2005). Acamprosate, which is FDA-approved for the treatment of alcohol use disorders, has been suggested to help cravings by reducing the cytokine-induced hyperglutamatergic state.

Furthermore, cognitive impairment may be a result of drug-induced neurotoxicity/ neurodegeneration, or a reduction in neurogenesis, all of which have been linked to neuroimmune signaling. For example, drug-induced microglial activation is associated with neuronal degeneration in both opioid and methamphetamine use (Raghavendra et al., 2004; Thomas and Kuhn, 2005). Furthermore, cytokine release is associated with a reduction of neurogenesis (Crews et al., 2011; Zou et al., 2012) and the blockade of NF-kB prevents against alcohol-induced reduction of neurogenesis (Crews et al., 2006).

There is strong evidence that alcohol use disorders are associated with a variety of cognitive deficits, including neurodegeneration in corticolimbic system (Crews and Nixon, 2009; Pfefferbaum et al., 1992; Obernier et al., 2002; Sullivan et al., 1995). Although this is associated with cytokine signaling and microglial activation, it remains unclear which comes first, or if the signaling is neurotoxic or neurotrophic (Marshall et al., 2013). Furthermore, there is evidence that alcohol-mediated dysregulation of prefrontal NF-kB contributes to changes in neuroplasticity seen in drug abuse (Okvist et al., 2007).

Neuroimmune activation may contribute to all three stages of drug abuse through a variety of mechanisms. The interaction between stress, depression, and cytokine signaling suggests that neuroimmune signaling is a promising avenue through which to target drug abuse therapy.

ALCOHOL AND NEUROIMMUNE SIGNALING

A growing body of literature has implicated neuroimmune signaling, including the neuroinflammatory response, in the mechanisms of neurobiological changes that promote unhealthy alcohol drinking behavior. For example, gene expression analyses of alcohol-preferring rodent strains have revealed candidate genes for high alcohol consumption which include many neuroimmune signaling genes (Mulligan et al., 2006; Kimpel et al., 2007; Tabakoff et al., 2008). Also, a gene expression study in humans has revealed that

the human alcoholic brain has changes in expression related to neuroimmune signaling and neurodegeneration when compared to controls (Liu et al., 2006).

Additionally, recent studies have revealed that alcohol can directly affect neuroimmune signaling, including an increase in pro-inflammatory signaling. For example, studies have found increases in several cytokines in several regions of the brain of mice after intragastric ethanol administration (Qin et al., 2008) or exposure of brain slices to ethanol (Zou and Crews, 2010), including MCP-1, TNFα, interleukin-1beta (IL-1β) proinflammatory proteases TACE and tissue plasminogen activator (tPA), and nicotinamide adenine dinucleotide phosphate oxidase (NOX), as well as reductions in anti-inflammatory cytokine IL-10. He and Crews (2008) found that ethanol increased cytokine levels and increased microglial infiltration in human alcoholic brains. Other studies have shown that ethanol increases inflammatory mediators in a rodent model of adolescent ethanol self-administration (Pascual et al., 2007) and a rodent model of chronic ethanol exposure (Valles et al., 2004).

These findings have helped fuel the theory that neuroimmune signaling can facilitate the development of the alcoholic phenotype and this theory has been supported by some recent studies. A recent study by Blednov et al. (2011) showed that the activation of inflammatory signaling with a single systemic injection of lipopolysaccharide produced a long-lasting increase in ethanol consumption in a 2-bottle choice paradigm in certain strains of mice at various time points after the injection, even up to 80 days after the

injection. Furthermore, the knockout of neuroimmune genes critical for the neuroimmune response, including many of the candidate genes from the studies previously mentioned, reduced alcohol consumption in animal models, including beta-2-microglobulin (B2m), cathepsin S (Ctss), cathepsin F (Ctsf), interleukin 1 receptor antagonist (Il1rn), CD14 molecule (Cd14), interleukin 6 (Il6), MCP1 (CCL2), CCR2, and CCL3 (Blednov et al., 2005; Blednov et al, 2011b). Understanding the role of specific neuroimmune molecules in the development of unhealthy drinking behavior would allow for the development of targeted neuroimmune modulators for therapeutic use.

MONOCYTE CHEMOATTRACTANT PROTEIN – 1 (MCP-1)

MCP-1 signaling is central to the neuroinflammatory response, with its main functions being to recruit microglia to sites of brain injury and, at higher concentrations, to regulate blood-brain barrier permeability and leukocyte infiltration (Piao et al., 2008; Stamatovic et al., 2005; Gunn et al., 1997). MCP-1 has been implicated in a variety of neuroinflammatory and neurodegenerative diseases, including brain ischemia, multiple sclerosis, traumatic brain injury, Alzheimer's Disease, and experimental autoimmune encephalomyelitis (Conductier et al., 2010).

In addition to providing the most potent chemotactic gradient (Sozzani et al., 1994), MCP-1 binds to its innate receptor (CCR2) on **neurons**, astrocytes, and activated microglia (Banisadr et al., 2002), endothelial cells (Stamatovic et al., 2005) and infiltrating

leukocytes (Cushing and Fogelman, 1991), to induce neuroimmune gene expression and a variety of cellular responses. In activated microglia, this results in the activation of the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), resulting in cellular motility and the induction of neuroimmune signaling genes (Figure 1.2; Piao et al., 2008). Astrocytes, neurons, infiltrating leukocytes, and activated microglia can also secrete MCP-1 in response to a variety of stimuli (for a review, see Semple et al., 2010). However, MCP-1 by itself cannot activate microglia, and microglia do not secrete MCP-1 until activated (Hinojosa et al., 2011; Flugel et al., 2001; Gourmala et al., 1997; Gunn et al., 1997).

The presence of CCR2 receptors on neurons is fitting given the role that neurons play in the neuroinflammatory responses as well as sickness behavior. MCP-1 receptors (CCR2) are found throughout the brain and are particularly dense in the prefrontal cortex, nucleus accumbens, amygdala, and ventral tegmental area, and include neuronal expression in many of these areas (Banisadr et al., 2002; Banisadr et al., 2005). MCP-1 is also secreted from many of those same brain regions as well (Banisadr et al., 2005b). Many of these brain regions are noteworthy because they are known to have a role in behavioral pathology, including drug abuse and addiction. The activation of CCR2 on GABA neurons has an allosteric inhibitory effect, where administration of MCP-1 alone has no effect on firing patterns, but causes a dose-dependent decrease in GABA-induced inward currents (Gosselin et al., 2005). Spinal cord neurons bathed in 10nm MCP-1 had a 50% reduction

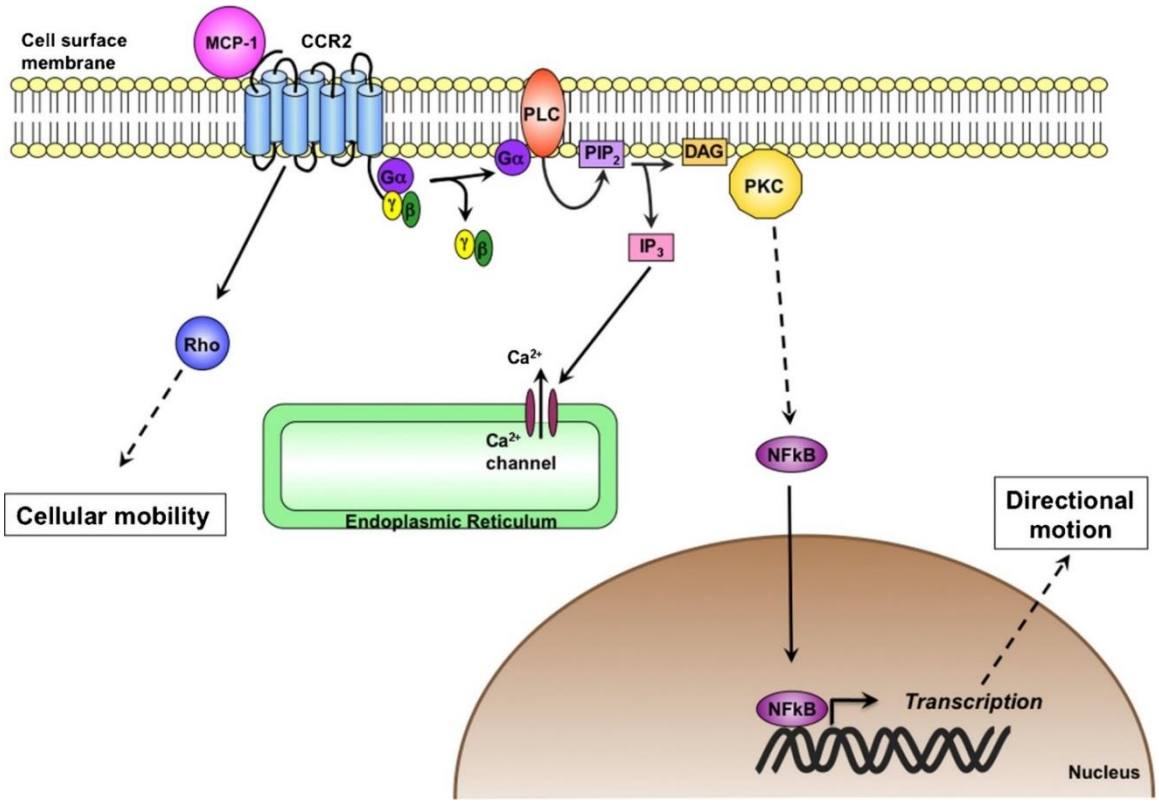

Figure 1.2: Overview of the intracellular cascade activated by CCR2.

All chemokine receptors are g-protein coupled receptors. CCR2 receptor activation results in the activation of protein kinase C, which in microglia, activates the transcription factor NF-kB. CCR2 activation in microglia results in the downstream effects of cellular mobility and directional motion. Taken from Melgarejo et al. (2009) with permission.

in current due to 100uM GABA, while 100nM MCP-1 nearly eliminated the current (Gosselin et al., 2005). Again, this could be particularly meaningful for interactions with drugs of abuse, especially those that act on GABA receptors, such as alcohol. Furthermore, MCP-1 receptors are located on dopamine neurons in the basal ganglia, which have a well-documented effect on motivated behaviors (Salamone and Correa 2002). CCR2 receptors are constitutively expressed on dopamine neurons (Banisadr et al., 2005), the activation of which leads to increases in phosphorylated tyrosine hydroxylase (Wakida et al., 2014) and increases in dopamine release (Guyon et al., 2009). There is some evidence that this is due to MCP-1 modulation of potassium channels (Guyon et al, 2009; Apartis et al, 2010; Wakida et al., 2014). However, it's also possible that MCP-1 leads to an increase in dopamine release through a well-known interaction between GABAergic interneurons and dopamine neurons in the VTA, where decreases in GABA release result in increases in dopamine release (Valenta et al., 2013). Others have shown that CRF can increase the presence of CCR2 on dopamine neurons, possibly through an interaction with TLR4 (June et al., 2015). Altogether, these data suggest that MCP-1 can influence motivation, reward, and anhedonia through modulation of neuronal activity, particularly through actions on neurons.

MCP-1 AND ALCOHOL

One cytokine in particular, monocyte chemoattractant protein 1 (MCP-1 or CCL2) and its receptor, CCR2, are of particular interest. There is evidence that MCP-1 promotes

ethanol-induced neurobiological changes. For example, brain injections of MCP-1 progressively exaggerate alcohol withdrawal-induced anxiety behavior (Breese et al., 2008). Also, there is evidence that alcohol directly increases MCP-1 signaling. Zou and Crews (2010) discovered a 1000% increase in MCP-1 over control animals in brain slices exposed to alcohol, MCP-1 concentrations were increased by 2-3 fold in a mice after intragastric administration of 5 g/kg ethanol for 8 days (Qin et al., 2008), and MCP-1 levels were increased by 2 to 3-fold in several regions of human alcoholic brains compared to controls (He and Crews, 2008). Furthermore, there is evidence that MCP-1 signaling modulates alcohol drinking behavior. MCP-1 receptor (CCR2) knockout mice had a robust reduction in alcohol consumption and a reduced preference for alcohol (Figure 1.3; Blednov et al., 2005).

HYPOTHESIS

Altogether, this data suggests that MCP-1 signaling is important in the development of ethanol drinking behavior of wild-type rodents, that MCP-1 signaling may accelerate the onset of alcohol dependence or increase the severity of alcohol dependence, and identifies MCP-1 and its receptor as potential target molecules for therapeutic intervention. The goal of my dissertation is to test the hypothesis that MCP-1-mediated neuroimmune signaling can influence alcohol self-administration. In particular, we theorize that enhancing MCP-1 signaling will accelerate the onset of drinking behavior and increase the severity of drinking behavior. Generally speaking, I accomplished this by

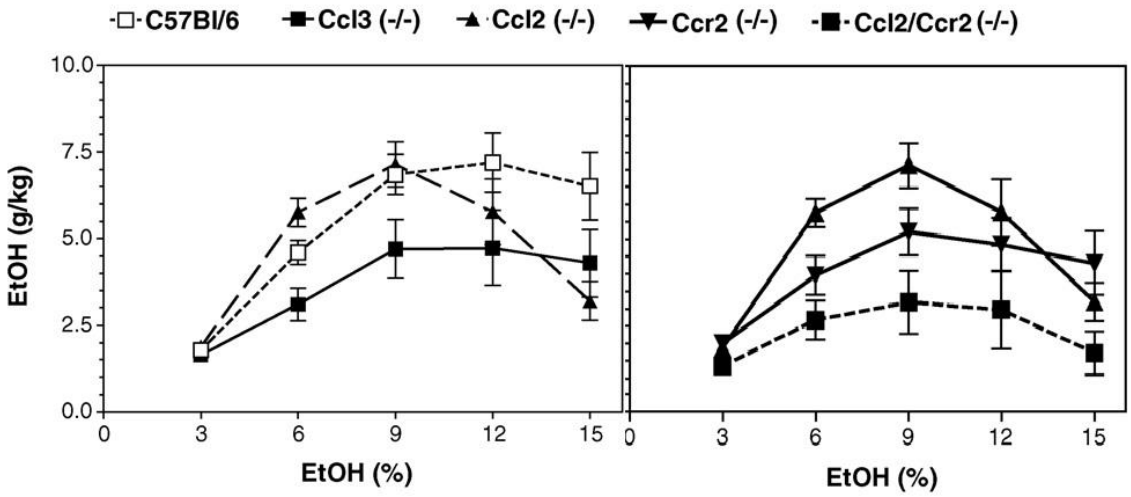

Figure 1.3: MCP-1 and CCR2 knockout mice drink less ethanol.

Escalating concentrations of ethanol were offered for 4 days at a time using a home-cage, 2 bottle choice design. In the left panel, wild type mice (C57BL/6J) are compared to CCL2 knockout mice and CCL3 knockout mice. ANOVA for wild type vs CCL2 knockout was not significant, but post-hoc analysis revealed a significance at 15% ethanol. In the right panel, double CCR2/CCL2 KO mice drank significantly less than either KO individually. The double-KO compared to wild-type in the left panel demonstrates the power of MCP-1 signaling in influencing ethanol intake. Adapted from Blednov et al. (2005) with permission.

increasing the amount of MCP-1 signaling in the brain and then measured drinking behavior. There are three phases of drinking behavior I investigated. First, I looked at the acquisition of drinking behavior (i.e. an animal that has never drunk before was given a chronic infusion of MCP-1 and I measured how fast the animal began to drink and how fast the drinking escalated). After a week or so, the animals generally stabilized their alcohol intake, and I measured if the MCP-1 infusion influenced the average daily intake during "stable" intake for several weeks. Third, I looked at how long the influence on drinking lasted after the MCP-1 infusion was stopped.

Chapter 2: Methods Overview

In order to keep the accepted manuscript with the MCP-1 experiments in an unmodified form (Chapter 3), I have included this chapter to describe methodology details that were not included in the manuscript.

MICROINJECTION OF MCP-1

MCP-1 signaling is complex and can lead to a variety of behavioral changes in the animal over time. There seem to be two distinct MCP-1 mediated effects on behavior after central administration; the acute transient depressive effects (Banisadr et al., 2002; Plata-Salaman and Borkoski, 1994) and the sustained behavioral effects (Breese et al., 2008). The study by Banisadr et al., (2002) found a decrease in locomotor activity 4 minutes after the MCP-1 injection, while Breese et al., (2008) tested locomotor activity several days later, after 5 days of ethanol administration, and found no effect. Loss of appetite was found 2 hours after microinjection but recovered by 24 hours (Plata-Salaman and Borkoski, 1994). Persistent effects include hyperalgesia and allodynia, which can last for 2-4 days (Baamonde et al., 2011; Tanaka et al., 2004; Dansereau et al., 2008), as well as chronic elevation in withdrawal-induced anxiety, measured 1-2 weeks later (Breese et al., 2008), and leukocyte infiltration, measured several days later (Stamatovic et al., 2005). Therefore, the timing of the delivery of MCP-1 are critical in regards to making accurate and meaningful measurements of their effect on operant self-administration. Furthermore, the

chronic nature of the experiment would require many stressful microinjections over several weeks, inviting another confounding variable and the likelihood that many rodents would be lost. Therefore, we plan to use an osmotic minipump, which will allow us to continuously perfuse very low volumes over extended amounts of time, with certain models of the Azlet® osmotic minipump capable of delivering 0.1 microliters per hour over 5 weeks. These pumps have been previously used to successfully administer chronic ICV MCP-1 (Stamatovic et al., 2005).

The ICV technique has been chosen due to the limited ability of MCP-1 to cross the blood brain barrier. ICV microinjections of cytokines, including MCP-1, and their antagonists have been shown to distribute into the brain parenchyma (Proeschold et al., 2002; Callewaere et al., 2006; Stamatovic et al., 2005). Unilateral ICV microinjections of cytokines induce brain-wide effects including glial activation and immune cell infiltration (Proeschold et al., 2002), and unilateral intracranial or ICV microinjections of MCP-1 using the osmotic minipump cause robust brain-wide effects including leukocyte infiltration (Stamatovic et al., 2005). Therefore, we plan to implant only one cannula and one osmotic minipump. Continuous delivery of MCP-1 or vehicle (ACSF) will continue while the animal recovers. Following the technique of Banisadr et al., (2002), the stereotaxic coordinates from Paxinos and Watson (1998) will be AP: – 0.9 mm, ML: 1.5 mm from bregma, DV: 2.5 mm from the skull surface.

The doses of MCP-1 were chosen based on its ability to stimulate persistent behavioral effects while not stimulating leukocyte infiltration or breakdown of the blood-brain barrier (Stamatovic et al., 2005). A dose of 20 ng to 100 ng of ICV MCP-1, administered as a bolus, has been used effectively by many researchers (Plata-Salaman and Borkoski, 1994; Banisadr et al., 2002; Breese et al., 2008; Baamonde et al., 2011; Tanaka et al., 2004; Dansereau et al., 2008). However, due to the difficulty in translating a bolus dose into a chronic infusion rate, we made sure to use a wide range of doses. Therefore, we will start with an osmotic delivery rate of 20 ng of MCP-1 per 24 hours and also include 100-fold higher and 100-fold lower doses. The threshold of blood-brain-barrier breakdown is 20-25 micrograms per day (Stamatovic et al., 2005).

SURGERY

Surgery will be planned so that animals will have a minimum of 4 days to recover before operant sessions begin. All instruments and materials are sterilized prior to use. Aseptic technique is used throughout the procedure. Anesthesia is carried out using isoflurane gas. Induction is at 5% and maintenance is at 2-3% (2 liter/min in O_2). After induction, the surgery site (top of animal's head) is shaved. Pre-incision, the surgery site is rough scrubbed with 10% povidone-iodine. The animal is secured in a stereotaxic apparatus using ear bars and a tooth hook to properly align the head for the stereotaxic surgery. A cut is made (approximately 1 inch long) on top of the skull. The skull is cleaned to expose surface landmarks. A small hole is drilled into the skull at the appropriate

position, based on anatomic coordinates, to place a stainless steel guide cannula above the desired structure. The cannula will be used as a chronic microinfusion tube, connected to an osmotic minipump (www.alzet.com) that will be implanted under the skin of the upper back. The minipump (approximate volume 200uL) will be pushed under the skin from the head to the upper back, so no additional cuts will be made. The pumps, cannula and tubing connecting the two arrive sterile but are safe to be re-sterilized through one of several procedures if necessary. No tether bolt, screws, or dental cement are necessary to install the guide cannula, only a small drop of super glue. This will allow for a quick surgery (<1hr) and leave a small wound and should facilitate healing. Sutures or staples are placed along the incisions as needed for proper healing. Bupivicaine will be administered intradermally and topically around the wound sites. Gentamicin and Neosporin ointment are applied topically, immediately and any time afterward for wound care.

Recovery is monitored continuously within the surgery suite until the animal is conscious. Water, soft baby cereal, and pellets are provided in the cage. The soft cereal is removed the following day. Body weight is monitored daily for 3-5 days after surgery, in addition to any day of testing. Our previous experience with this technique suggests the procedure is well tolerated by the animals. Any new wounds or worsening wounds will be reported to the vet staff and subsequent wound care will be determined by the veterinary staff. The rat will be monitored and if the microinjector becomes loose, the rodent will be euthanized. Additionally, lack of gain of body weight after surgery, lack of normal grooming and locomotion, redness or discharge around eyes, lack of eating or drinking

would indicate that the rat is not recovering normally and the rat will be promptly euthanized.

JUSTIFICATION FOR THE USE OF LONG-EVANS RATS

The use of whole animals (rats) is required for these studies because some of the mechanisms of ethanol are due to interactions between neurons and brain regions which will only be operative in the whole animal. Rats exhibit most of the pharmacological effects of ethanol, including self-administration, intoxication, persistence of ethanol-seeking, and, therefore, represent a useful model of human drinking. The interactions we are studying cannot be modeled using tissue culture or invertebrates. The size of the brain also allows precise placement of microinjectors and probes to investigate specific brain regions that may contribute to the mechanisms that we study. A smaller species such as a mouse would make precise placements very difficult. We do not need to use larger animals because we have been able to achieve good results with rats. We do not yet have a clear understanding of the molecular and cellular effects of ethanol on brain function to generate a computer model. Human epidemiological studies cannot be used to understand the effects of changes in neurochemistry produced by ethanol consumption.

Male Long-Evans rodents (Rattus norvegicus) were used in all experiments since they display reliable ethanol self-administration at intoxicating levels using the models proposed. A priori power analysis determined that to observe an effect size of 20%, with

power set at 0.80, we will need a minimum of 10 animals per group. We chose 12 per group because we expect some attrition due to incorrect probe placements or rats that do not drink enough alcohol to be used for the experiment. Lower numbers of animals will give a high probability of coming to a false conclusion regarding the results

The Animal Resources Center maintains a full-time staff for the care of the animals. Drs. Glen Otto and Kathleen Roellich are the University's full-time veterinarians. They are both available 24 hours a day for help with animal care, and they routinely participate in the training of investigators. Our lab also personally monitors animals daily for health. We very closely monitor the recovery of animals that have had surgery. If we encounter any problems, we contact Drs. Otto or Roellich who advise us on the best course of action.

All procedures will be carried out in strict accordance to the NIH Guide for the Care and Use of Laboratory Animals and will be approved by the Institutional Animal Care and Use Committee of the University of Texas at Austin. The Animal Resources Center at the University of Texas at Austin is AAALAC certified and has a full-time animal care staff and 24-hour veterinary care. The neurochemical measurements which are proposed are especially susceptible to stress in the animals and because of this and ethical considerations, stress will be minimized. Rodents will be monitored several times a day by animal care staff as well as me and the researchers helping me. All personnel have been trained in general animal and experimental guidelines through coursework provided by the American Association for Laboratory Animal Science (AALAS), including "Rodent

Handling Techniques" and "Pain Recognition and Alleviation." Any signs of distress will be immediately reported to the veterinary staff and proper action taken. All surgeries will be performed with proper anesthesia and animals will be monitored constantly.

IACUC approved standard protocols, and NIH and AVMA guidelines for euthanasia will be followed. After operant testing is complete, animals will be sacrificed using Euthasol ® solution (pentobarbital 150mg/kg). This method has been chosen because it causes no distress to the animal other than the minimal transient pain of intraperitoneal injection. However, under most circumstances, the animal will be injected with Euthasol after anesthesia induction using isoflurane.

Chapter 3: Chronic intracerebroventricular infusion of monocyte chemoattractant protein – 1 leads to a persistent increase in sweetened ethanol consumption during operant self-administration but does not influence sucrose consumption in Long-Evans rats.

This work was accepted for publication in *Alcoholism: Clinical and Experimental Research* on October 12, 2015, under the same title, by Valenta JP and Gonzales RA. It is slated to be published in the January, 2016 issue.

ABSTRACT

Background: Among the evidence implicating neuroimmune signaling in alcohol use disorders are increased levels of the chemokine monocyte chemoattractant protein-1 (MCP-1) in the brains of human alcoholics and animal models of alcohol abuse. However, it is not known whether neuroimmune signaling can directly increase ethanol consumption, and whether MCP-1 is involved in that mechanism. We designed experiments to determine if MCP-1 signaling itself is sufficient to accelerate or increase ethanol consumption. Our hypothesis was that increasing MCP-1 signaling by directly infusing it into the brain would increase operant ethanol self-administration.

Methods: We implanted osmotic minipumps to chronically infuse either one of several doses of MCP-1 or vehicle into the cerebral ventricles (ICV) of Long-Evans rats and then tested them in the operant self-administration of a sweetened ethanol solution for 8 weeks.

Results: There was a significant interaction between dose of MCP-1 and sweetened ethanol consumed across the first 4 weeks (while pumps were flowing) and across the 8-week experiment. Animals receiving the highest dose of MCP-1 (2 μg/day) were the highest consumers of ethanol during weeks 3 through 8. MCP-1 did not influence the acquisition of self-administration (measured across the first 5 days), the motivation to consume ethanol (time to lever press or progressive ratio), withdrawal-induced anxiety, or the consumption of sucrose alone.

Conclusion: We provide novel evidence that neuroimmune signaling can directly increase chronic operant ethanol self-administration, and that this increase persists beyond the administration of the cytokine. These data suggest that ethanol-induced increases in MCP-1, or increases in MCP-1 due to various other neuroimmune mechanisms, may further promote ethanol consumption. Continued research into this mechanism, particularly using models of alcohol dependence, will help determine if targeting MCP-1 signaling has therapeutic potential in the treatment of alcohol use disorders.

Key Words: dose-response, NF-κB, neuroinflammation, neuromodulation, dopamine neurons

INTRODUCTION

Recent research has implicated neuroimmune signaling in the neurobiological changes that promote unhealthy alcohol drinking behavior (for a review, see Crews et al., 2011). A variety of experiments have revealed that a specific chemokine, monocyte

chemoattractant protein-1 (MCP-1, or CCL2), and its receptor, CCR2, are particularly significant. For example, Zou and Crews (2010) discovered a 1000% increase in MCP-1 in brain slice cultures treated with ethanol, relative to controls. Brain MCP-1 concentrations were increased 2-3 fold in mouse models of sub-chronic or chronic ethanol exposure (Qin et al. 2008, Pascual et al., 2015), in a rat model of chronic ethanol exposure (Ehrlich et al., 2012), as well as in human alcoholic brains post-mortem (He and Crews, 2008). Ehrlich and colleagues (2012) found elevated cortical MCP-1 in rats on a 20% ethanol liquid-only diet for 12 months. Furthermore, manipulating MCP-1 signaling can influence ethanol self-administration in animal models. For example, MCP-1 ligand, receptor, and combined ligand and receptor knockout mice had a robust reduction in ethanol consumption and preference (Blednov et al., 2005). More recently, siRNA-mediated knockdown of neuronal MCP-1 expression in the ventral tegmental area or central amygdala resulted in marked decreases in operant responding for ethanol (June et al., 2015). Additionally, June et al. (2015) found evidence that corticotropin-releasing factor (CRF) modulates MCP-1 expression in neurons. Interestingly, MCP-1 has been previously shown to enhance dopamine neurotransmission through modulation of potassium channels (Guyon et al, 2009; Apartis et al, 2010; Wakida et al., 2014), leading the authors of June et al. (2015) to hypothesize that CRF signaling regulates excessive alcohol drinking through the modulation of MCP-1 expression on dopamine neurons. Altogether, these data suggest that ethanol-induced increases in MCP-1 may facilitate increased ethanol consumption. Experimental manipulation to determine if MCP-1 signaling itself can directly increase ethanol consumption was warranted.

Our hypothesis was that increasing MCP-1 signaling by directly infusing it into the brain would increase the operant self-administration of sweetened ethanol. We designed an experiment that would allow us to independently examine the effect of MCP-1 on several distinct components of ethanol self-administration, including acquisition, motivation, and consumption. We also examined withdrawal-induced anxiety. We implanted subcutaneous osmotic minipumps connected to intracranial cannulae to chronically infuse a wide range of concentrations of MCP-1 into the cerebral ventricles (ICV) of Long-Evans rats, using doses that are substantially below those required to trigger neuroinflammatory mechanisms such as BBB breakdown or leukocyte infiltration (Stamatovic et al., 2005). We provide novel evidence that neuroimmune signaling can directly increase chronic operant ethanol self-administration, and that this increase persists beyond the administration of the cytokine.

MATERIALS AND METHODS

Timeline

During the 1st week, animals were acclimated and handled. During the 2nd week, animals were trained to lever press. During the 3rd week, MCP-1 was reconstituted and osmotic minipumps were filled and surgically implanted for ICV delivery of MCP-1. During weeks 4 through 11 (8 weeks), animals performed daily operant self-administration sessions of a sucrose-sweetened ethanol solution (Monday-Friday). Pumps delivered

MCP-1 continuously for 5 weeks and self-administration started during the 2nd week of delivery. Progressive ratio and withdrawal-induced anxiety tests were performed on the Sunday at the end of the 4th week of self-administration (on the last day of pump delivery of MCP-1). The self-administration of a sucrose solution without ethanol was performed in a separate group of animals and followed this timeline except that the experiment was stopped after 4 weeks of self-administration.

Animals

We used male Long-Evans rats (Charles River Laboratories, Wilmington, MA) weighing an average of 190 grams upon arrival and 350 grams on the first day of ethanol self-administration. The rats were dual-housed at 25 °C on a 12-h light/dark schedule (lights on 7 am to 7 pm) with ad libitum access to food and water in an AAALAC-accredited facility. All procedures were carried out in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of Texas at Austin.

Care was given to minimize stress to the animals. Animals were dual-housed immediately upon arrival and throughout the experiment. Rats were handled by every experimenter for at least 4 days prior to operant training for a total of 15-20 minutes each day. Each cage had a nylabone for enrichment throughout the study. Bedding was made from wood chips and was replaced weekly. The animal housing room is only occupied by rats used by the Gonzales lab and is adjacent to the room with operant chambers. Animals

are carried from their home cage through one open door directly to the operant chambers. Operant chambers are cleaned daily. The two rooms are isolated from other traffic and noises.

Operant Training

Animals were water deprived for 16-22 hours per day and trained to lever press for a 10% sucrose solution using an FR1 schedule of reinforcement. Self-administration took place in standard operant chambers (Med Associates Inc., St. Albans, VT). Each chamber contained a single, retractable lever on the left side (2 cm above the grid floor). Each time the animal pressed the lever, a retractable drinking spout entered the chamber on the right side of the same wall (5 cm above the grid floor). An interior chamber light and a sound-attenuating fan were activated with the start of each operant session. Operant chamber components were controlled by a personal computer running MEDPC software (Med Associates Inc., St. Albans, VT). Initially, 1-hour sessions were used, with session duration declining to 40 minutes and then 20 minutes for individual animals as they made progress. All rats were trained to lever press successfully within 5 days, with 20-minute sessions occurring during day 5 (or sooner).

MCP-1

Recombinant rat MCP-1 (Peprotech, Rocky Hill, NJ) was dissolved in dH2O and then diluted in concentrated artificial cerebro-spinal fluid (aCSF) to reach a standard final concentration of 149 mM NaCl, 2.8 mM KCl, 1.2 mM MgCl2,1.2 mM CaCl2, 0.42 mM ascorbic acid, 5.4 mM D-glucose, and 1% rat serum albumin (Sigma-Aldrich, St. Louis, MO). The solution was then serially diluted in aCSF (with final salt and albumin concentrations) to reach desired MCP-1 concentrations. Alzet osmotic minipumps (model 1004) and brain infusion cannulae ("kit 2", 28 ga) were obtained from Durect Corporation (Cupertino, CA). Pumps were filled with a specific concentration of MCP-1 adjusted to each lot's individual fill volume and flow rate to yield a final dose of either 0.2 ng/day, 20 ng/day, 2000 ng/day, or vehicle control. An additional group of 2 ng/day pumps were used for the ELISA experiment. The osmotic pumps used were designed to flow at a constant rate of 0.12 μL/hr for 34.5-35.9 days with a mean fill volume of approximately 100 μL, leading to an MCP-1 concentration of approximately 54 μM for the 2000 ng/day dose. MCP-1 was reconstituted and pumps were filled on Mondays and primed in sterile saline at 37°C to ensure reliable flow prior to implantation (which occurred on Tuesdays and Wednesdays), and should have reliably stopped flowing on the 35th day, according to the manufacturer.

The dose of MCP-1 was chosen based on the ability of an acute ICV dose of 20 – 100 ng to induce persistent behavioral effects (Plata-Salaman and Borkoski, 1994; Banisadr et al., 2002; Breese et al., 2008). Based on those experiments, we centered on the

delivery of 20 ng over a 24-hour period and then included 100-fold higher and 100-fold lower doses (which do not compromise the blood-brain barrier or lead to leukocyte infiltration when administered ICV (see discussion)). The chronic infusion method was chosen due to the propensity of the interaction between ethanol and neuroimmune signaling to be chronic in nature. We chose the ICV method to simulate the presence of brain-induced MCP-1 and the method's ability to target the whole brain with molecules that don't readily cross the blood-brain barrier (Dzenko et al., 2001). Evidence shows that ICV administration of MCP-1 or other cytokines distributes throughout the brain to initiate receptor-mediated effects (for MCP-1-specific data, see Stamatovic et al., 2005).

Surgery

The cannula tip was aimed at the left lateral ventricle using the following coordinates relative to bregma (mm): anterior/posterior -0.60, medial/lateral +1.50, and dorsal/ventral -3.80, and was then glued to the skull using cyanoacrylate adhesive. The pump was implanted subcutaneously in a pocket just under the skin which was created by sliding needle holders through a head incision to the mid upper-back region. A 5 cm polyethylene tube connected the pump to the L-shaped cannula. The skin was sutured over the cannula once the tab for stereotaxic placement was removed. Bupivicaine (Hospira, Inc., Lake Forest, IL) was administered intradermally, and both bupivacaine and gentamicin (APP Pharmaceuticals, LLC, Schaumburg, IL) were dripped into the wound (2 mg/kg each, in total).

Self-administration

Animals drank either a solution of 10% sucrose (w/v) alone or a solution with both 10% sucrose (w/v) and 10% ethanol (v/v). The solutions were made using 95 % ethanol (Aaper Alcohol and Chemical Co., Shelbyville, KY) and molecular biology grade D-sucrose (Fisher, Hampton, New Hampshire) dissolved in tap water.

Animals began self-administration the week after surgery (5-6 days after implantation). Animals self-administered Monday through Friday for 8 weeks, of which pumps delivered MPC-1 during the first 4 weeks. Self-administration sessions occurred between 5 and 8 hours into the light cycle. Due to the presence of sucrose, intake levels stabilize within a few days. We defined acquisition as the intake values across the first 5 days. The self-administration of 10% sucrose without ethanol was measured in a distinct group of animals and followed these methods, except limited to just the first 4 weeks of self-administration and the highest dose of MCP-1 (2000 ng/day) and controls.

To capture whether MCP-1 influenced the motivation to gain access to ethanol independent of the quantity consumed, we chose an appetitive-consummatory model of self-administration. The appetitive phase required the animal to press a lever 4 times in order to gain access to the ethanol solution, and the time taken to reach this response requirement was recorded. This phase was followed by the consummatory phase, which consisted of 20 minutes of access to the ethanol solution, and the amount of ethanol consumed was recorded. If 20 minutes passed without the animal pressing 4 times, access to the ethanol was automatically given. Ethanol was contained in a retractable sipper tube

with a 50 mL conical vial. Drippage was collected in a weigh boat, and both the vial/tube and the dish were weighed before and after the session to the hundredths of an mL.

As a secondary test of motivation, a progressive ratio test was administered on the final day of pump flow (the Sunday of the 5th week). We followed the model of Walker and Koob (2007) and used the following schedule of reinforcement: 2,2,3,3,4,4,5,5,7,7,9,9,11,11,13,13,15,15,18,18. The amount of ethanol consumed and the break point reached were measured.

Since MCP-1 is an inflammatory molecule with unknown effects during chronic administration, animals were closely monitored for illness, including body weight and locomotor activity. Locomotor activity was only recorded during the 20 minutes of ethanol access. Seven of 8 chambers had infrared sensors to measure locomotor activity.

Withdrawal-induced anxiety

Six to 8 hours after the progressive ratio session, animals were tested for withdrawal-induced anxiety by measuring the seconds of social interaction they initiated with an unfamiliar animal with a matched dose of MCP-1 over a 5 minute period. Generally, interaction was defined as contact directed at the other animal (sniffing, grooming, crawling, fighting, etc) and took place in a square open field (60 x 60 cm2, with 16 squares marked on the floor for assessing locomotor activity) and under low-lighting. A minimum of two observers, each blind to the treatment conditions, independently scored social interaction and locomotor activity.

Enzyme-Linked Immunosorbent Assay (ELISA)

The stability of MCP-1 under the experimental conditions was unknown. In a separate experiment, rat MCP-1 ELISA kits (Life Technologies, Grand Island, NY) were used to determine the stability of MCP-1 across the infusion duration. We tested the concentration of MCP-1 in the pumps of animals 14 and 28 days after MCP-1 was reconstituted and then diluted to a 2 ng/day dose (after 1 and 3 weeks of drinking, respectively). Pumps were taken from drinking animals that were sacrificed at the day of testing. Pumps were cut open and contents were quantified for MCP-1 according to the manufacturer's instructions.

Histology

After the experiment, animals were sacrificed using CO2 and then decapitated. Brains were extracted and put into 10% formalin for 3 days before being sliced and immediately examined under a microscope to determine cannula placement using Paxinos and Watson (2007).

Data analysis

Daily values were averaged for each week for each animal. A repeated measures ANOVA with a type I error set to $P<0.05$ was conducted across 4-week and 8-week data

sets (the first four weeks while pumps were flowing, the last four weeks while pumps were stopped, and the eight week combined set), or the first 5 days for the acquisition analysis. A one-way ANOVA was used for one-time experiments (progressive ratio and anxiety). Values are reported as mean ± SEM.

RESULTS

MCP-1 did not increase the operant self-administration of sucrose alone.

Chronic ICV infusion of MCP-1 started 1 week before the initiation of self-administration and continued for 4 weeks of self-administration, for a total of 5 weeks of infusion. MCP-1 did not significantly influence the self-administration of 10% sucrose solution (Figure 2.1, $F_{3,39}$=0.04, P=0.99, dose x time interaction, n=7 for controls and n=8 for MCP-1 group). Only the highest dose of MCP-1 (2000 ng/day) was tested.

MCP-1 did not influence the acquisition of operant self-administration of sweetened ethanol.

Chronic ICV infusion of MCP-1 started 1 week before the initiation of self-administration and continued through the acquisition period (5 days). ICV infusion of

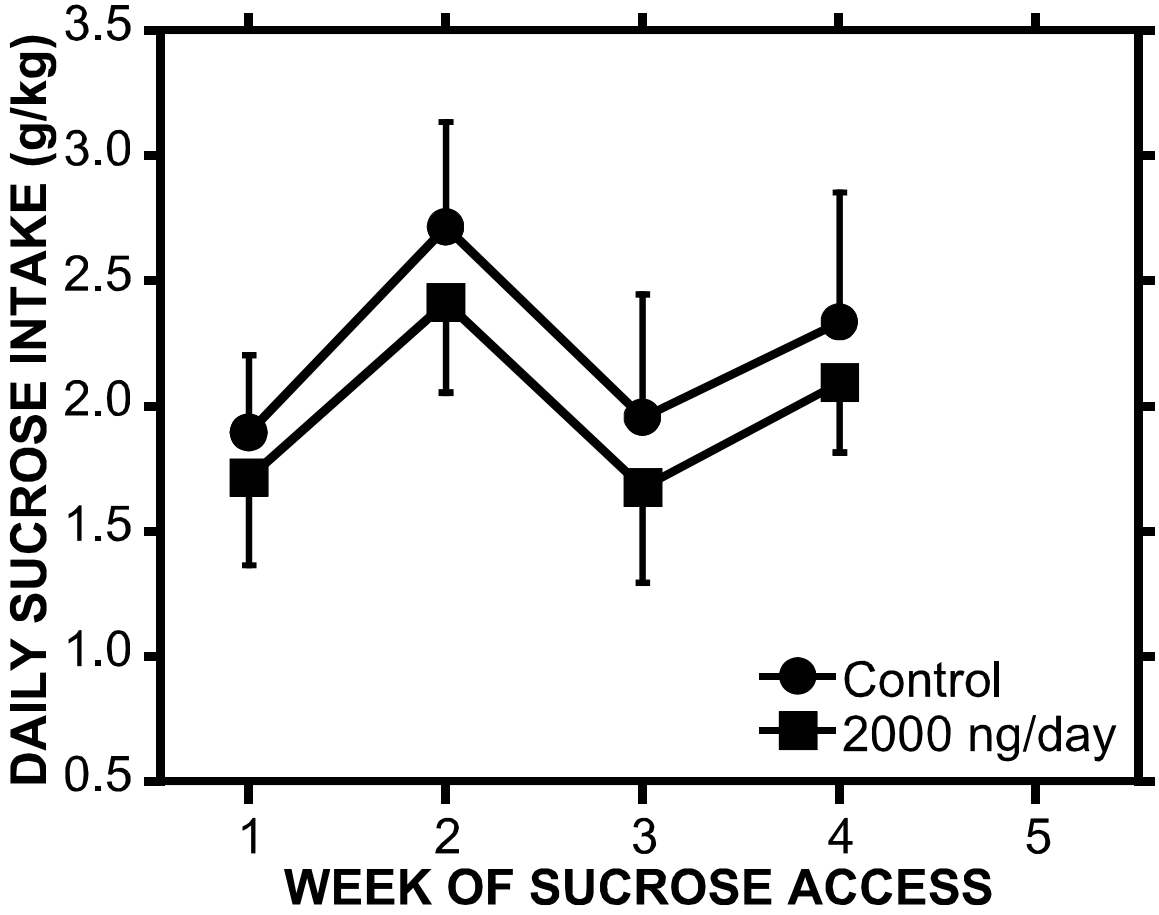

Figure 2.1: The effect of MCP-1 on the self-administration of sucrose.

MCP-1 was administered using subcutaneous osmotic minipumps over 5 weeks (1 week of recovery followed by 4 weeks of self-administration with pumps flowing) followed by 4 weeks of self-administration without flow. MCP-1 did not influence the self-administration of sucrose alone (P=0.99, n=7 for controls and n=8 for MCP-1 group).

MCP-1 had no effect on the acquisition of operant self-administration of sweetened ethanol, measured during the first 5 days (Figure 2.2, $F_{12,156}$=1.40, P=0.17 dose x time interaction, N=11/10/11/11 for control/0.2/20/2000 ng/day doses respectively).

MCP-1 increased consumption during chronic operant self-administration of sweetened ethanol.

Chronic ICV infusion of MCP-1 started 1 week before the initiation of self-administration and continued during 4 weeks of self-administration, for a total of 5 weeks of infusion. Self-administration took place across 8 weeks, the first 4 of which MCP-1 was being delivered. MCP-1 increased the self-administration of sweetened ethanol over 8 weeks with animals receiving the highest dose of MCP-1 (2 μg/day) drinking the most ethanol on average during weeks 3 through 8 (Figure 2.3/2.4, $F_{21,168}$ =1.65, P=0.04), dose x time interaction, n=7/6/7/8 for control/0.2/20/2000 ng/day doses respectively). The effect was also significant for the first 4 weeks of self-administration alone, while the pumps were flowing ($F_{9,72}$ =2.33, P=0.02, dose x time interaction). The effect was not significant when analyzed over the final 4-week period ($F_{9,72}$=1.566, P=0.14, dose x time interaction). Of the 43 animals used for the acquisition experiment, 28 were used for the chronic self-administration analyses (12 animals had pumps that stopped prior to 4 weeks, 2 animals were lost due to fighting and 1 due to infection).

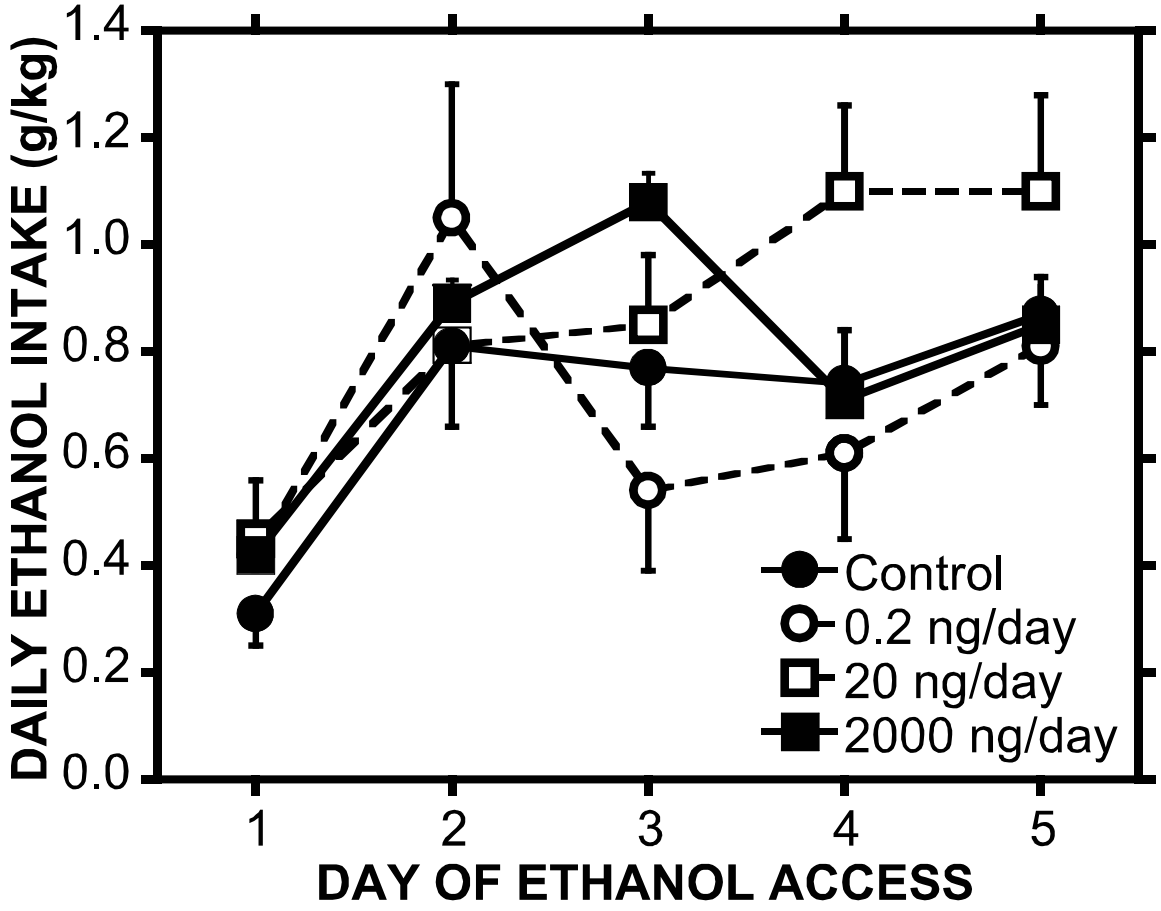

Figure 2.2: The effect of MCP-1 on the acquisition of ethanol intake.

ICV infusion of MCP-1 had no effect on the first 5 days of operant self-administration of sweetened ethanol (P=0.17, N=11/10/11/11 for control/0.2/20/2000 ng/day doses respectively). MCP-1 infusion began one week prior to the initiation of self-administration.

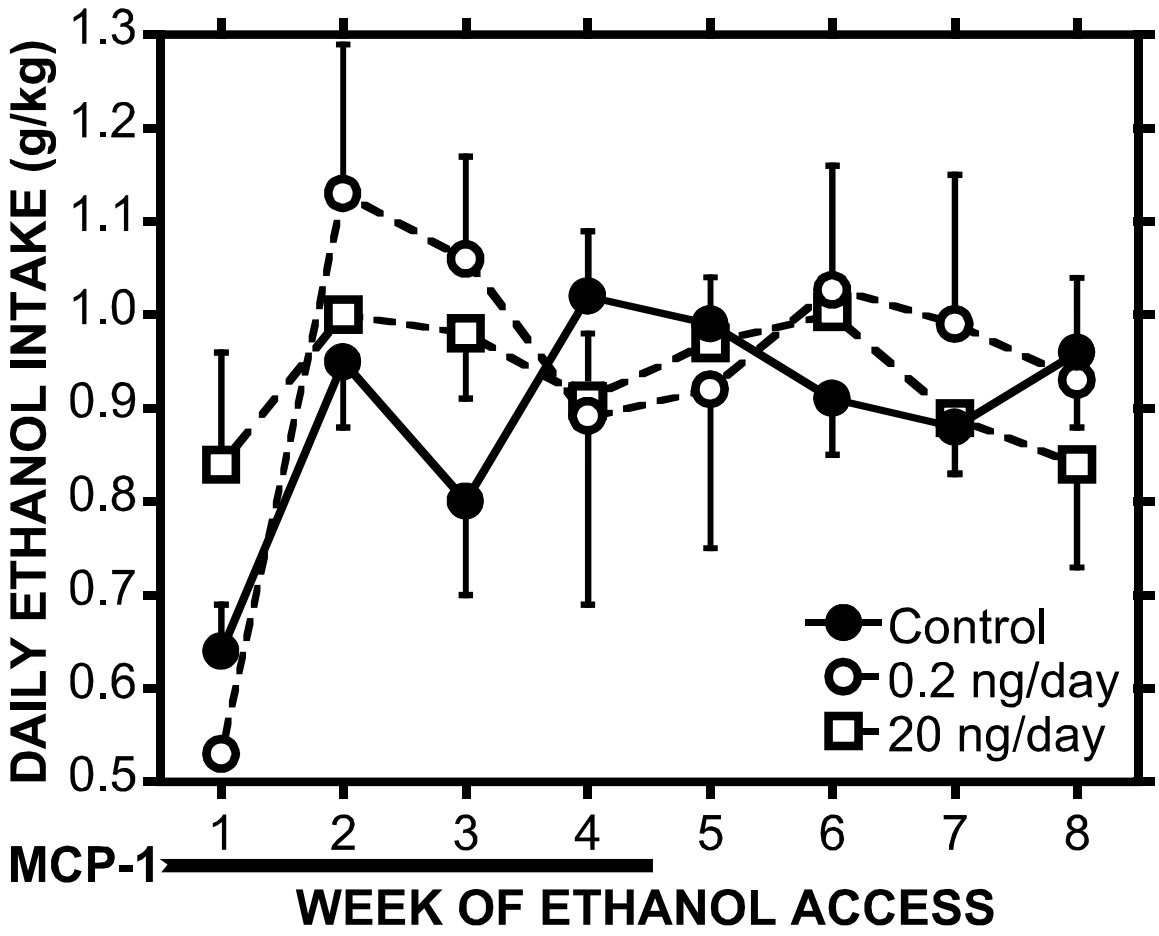

Figure 2.3: The effect of MCP-1 on the self-administration of sweetened ethanol.

MCP-1 increased the self-administration of sweetened ethanol over 8 weeks (P=0.04, n=7/6/7/8 for control/0.2/20/2000 ng/day doses respectively). The data is split across 2 graphs for clarity. The effect was also significant for the first four weeks of self-administration alone, while pumps were flowing (P=0.02). MCP-1 did not influence drinking over the final four-week period (P=0.14). For clarity, not all error bars are included. The black bar indicates MCP-1 infusion, which began one week prior to the initiation of self-administration.

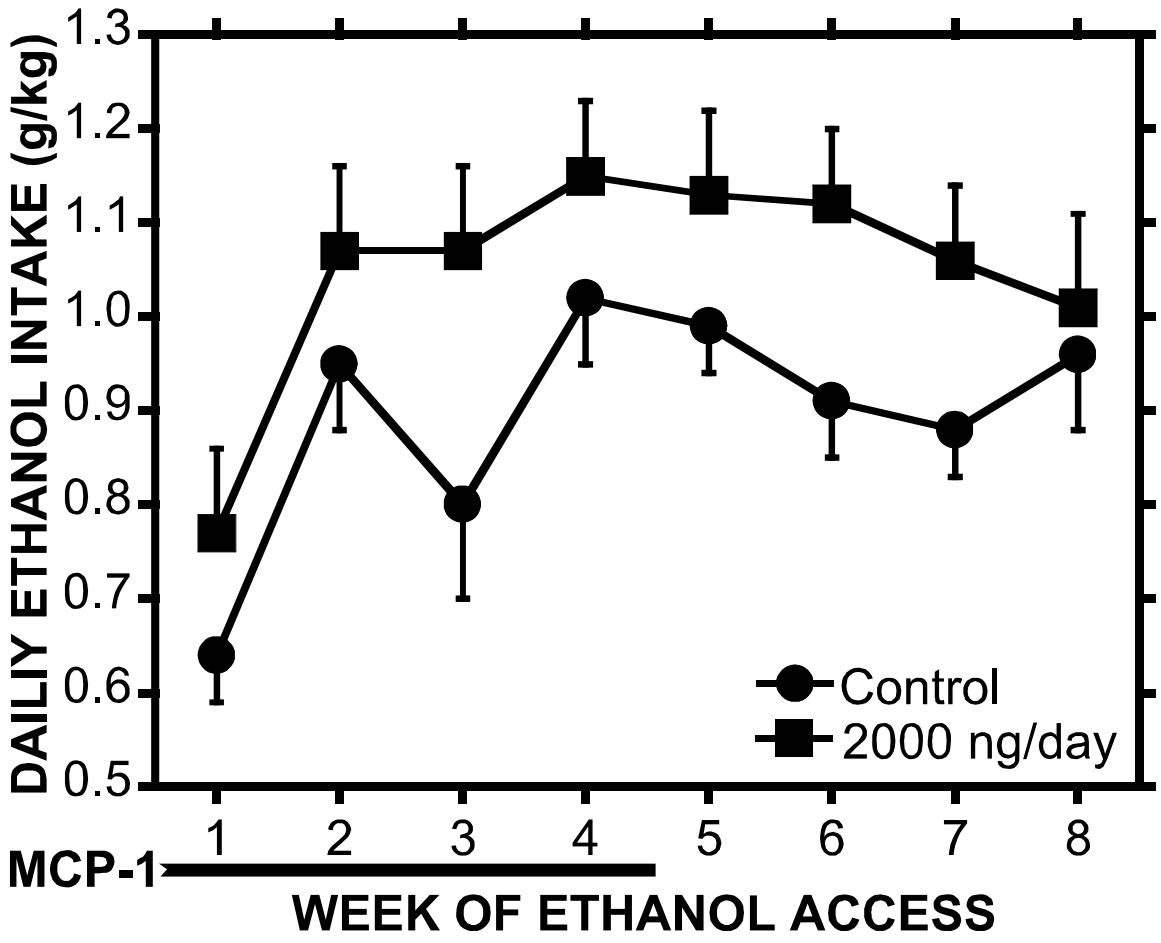

Figure 2.4: The effect of MCP-1 on the self-administration of sweetened ethanol.

MCP-1 increased the self-administration of sweetened ethanol over 8 weeks (P=0.04, n=7/6/7/8 for control/0.2/20/2000 ng/day doses respectively). The data is split across 2 graphs for clarity. The effect was also significant for the first four weeks of self-administration alone, while pumps were flowing (P=0.02). MCP-1 did not influence drinking over the final four-week period (P=0.14). For clarity, not all error bars are included. The black bar indicates MCP-1 infusion, which began one week prior to the initiation of self-administration.

MCP-1 did not influence health-related measures.

MCP-1 had no effect on the body weight of the animals ($F_{9,72}$=0.85, P=0.58, dose x time interaction, n=7/6/7/8 for control/0.2/20/2000 ng/day doses respectively) or locomotor activity ($F_{9,60}$=0.85, P=0.58, dose x time interaction, n=6/5/6/7 for control/0.2/20/2000 ng/day doses respectively) across the first 4 weeks of drinking. A summary of the data averaged across the first 4 weeks of drinking is shown in Figure 2.5 and Figure 2.6 for the body weight and locomotor activity, respectively. There was also no effect on % baseline body weight or any of the 8-week analyses (data not shown). No other overt signs of sickness were detected throughout the experiments.

MCP-1 did not increase the motivation to consume sweetened ethanol.

There was no effect on the time to reach the response requirement of 4 lever presses to gain access to the ethanol solution across the four weeks of drinking ($F_{9,72}$=0.49, P=0.88, dose x time interaction, n=7/6/7/8 for control/0.2/20/2000 ng/day doses respectively). A summary of the data averaged across the first 4 weeks of drinking is shown in Figure 2.7. There was also no effect across 8 weeks (data not shown). Animals that took longer than 2 standard deviations from the mean (266 seconds / 4.43 min) during a particular day were removed from the analysis (20 instances out of 1120, or 1.8%, across all 8 weeks). These exclusions were distributed across doses, with 6/5/7/2 exclusions for control/0.2/20/2000 ng/day doses respectively.

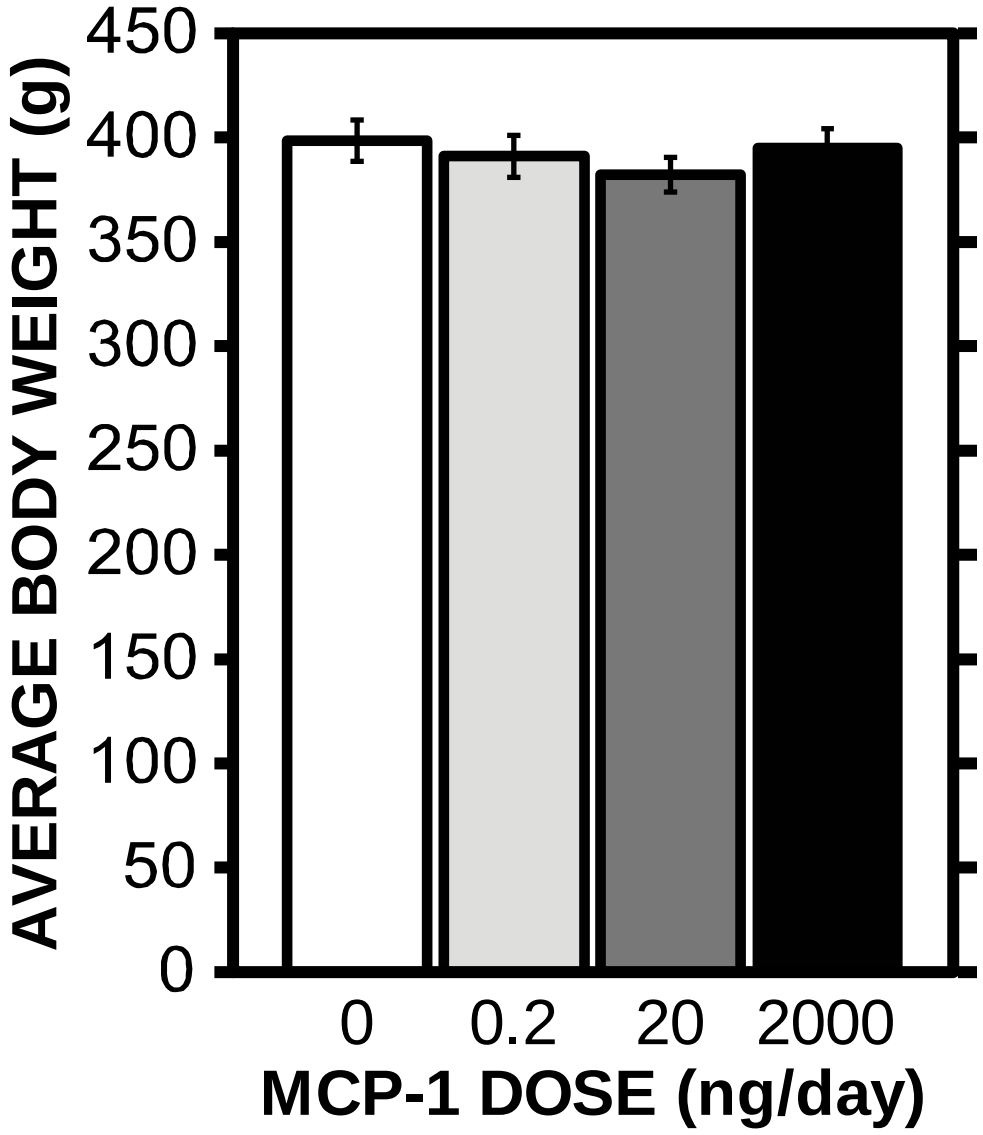

Figure 2.5: The effect of MCP-1 on body weight.

Chronic ICV infusion of MCP-1 had no effect on the body weight of the animals across 4 weeks of drinking (P=0.58, n=7/6/7/8 for control/0.2/20/2000 ng/day doses respectively). A summary of the data averaged across the first 4 weeks of drinking is shown.

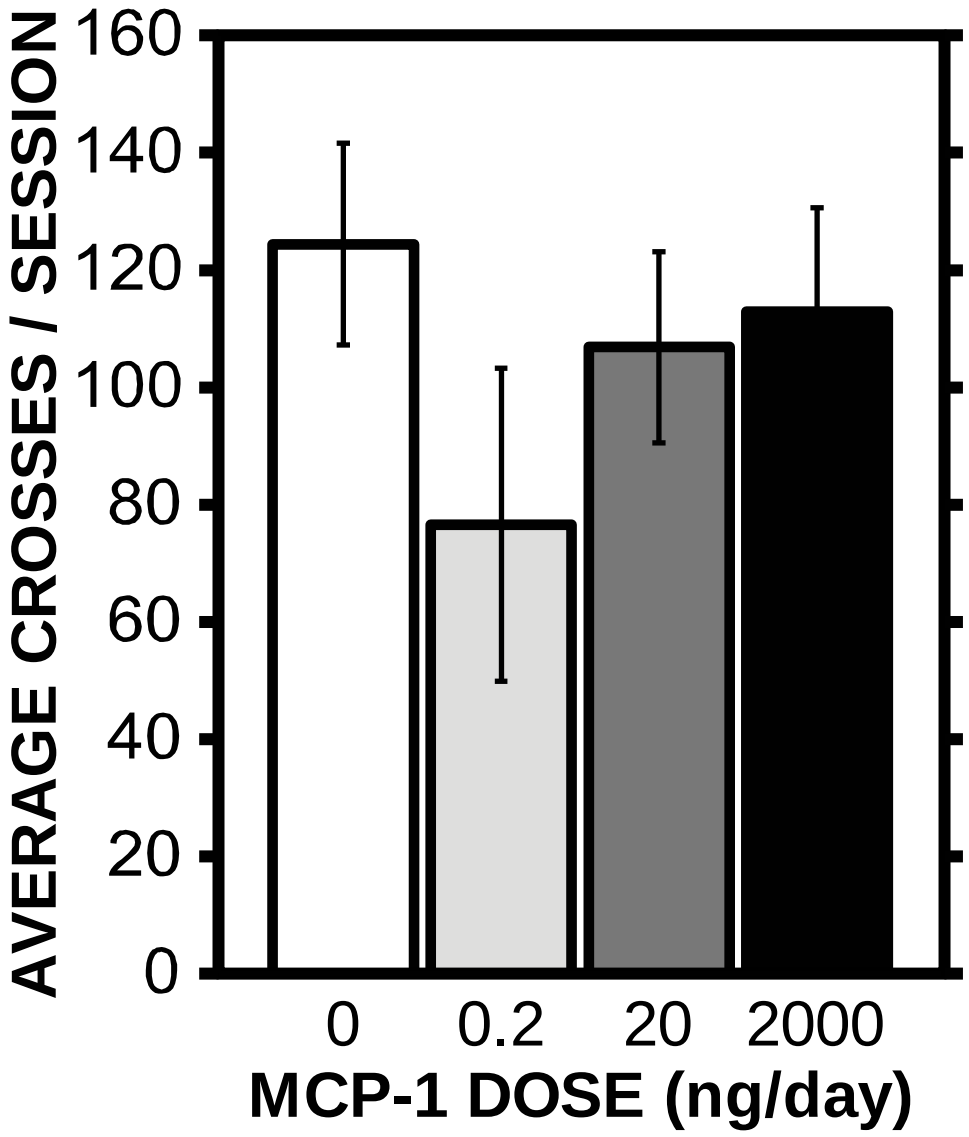

Figure 2.6: The effect of MCP-1 on locomotor activity.

Chronic ICV infusion of MCP-1 had no effect on locomotor activity during drinking sessions (P=0.58, n=6/5/6/7 for control/0.2/20/2000 ng/day doses respectively). A summary of the data averaged across the first 4 weeks of drinking is shown.

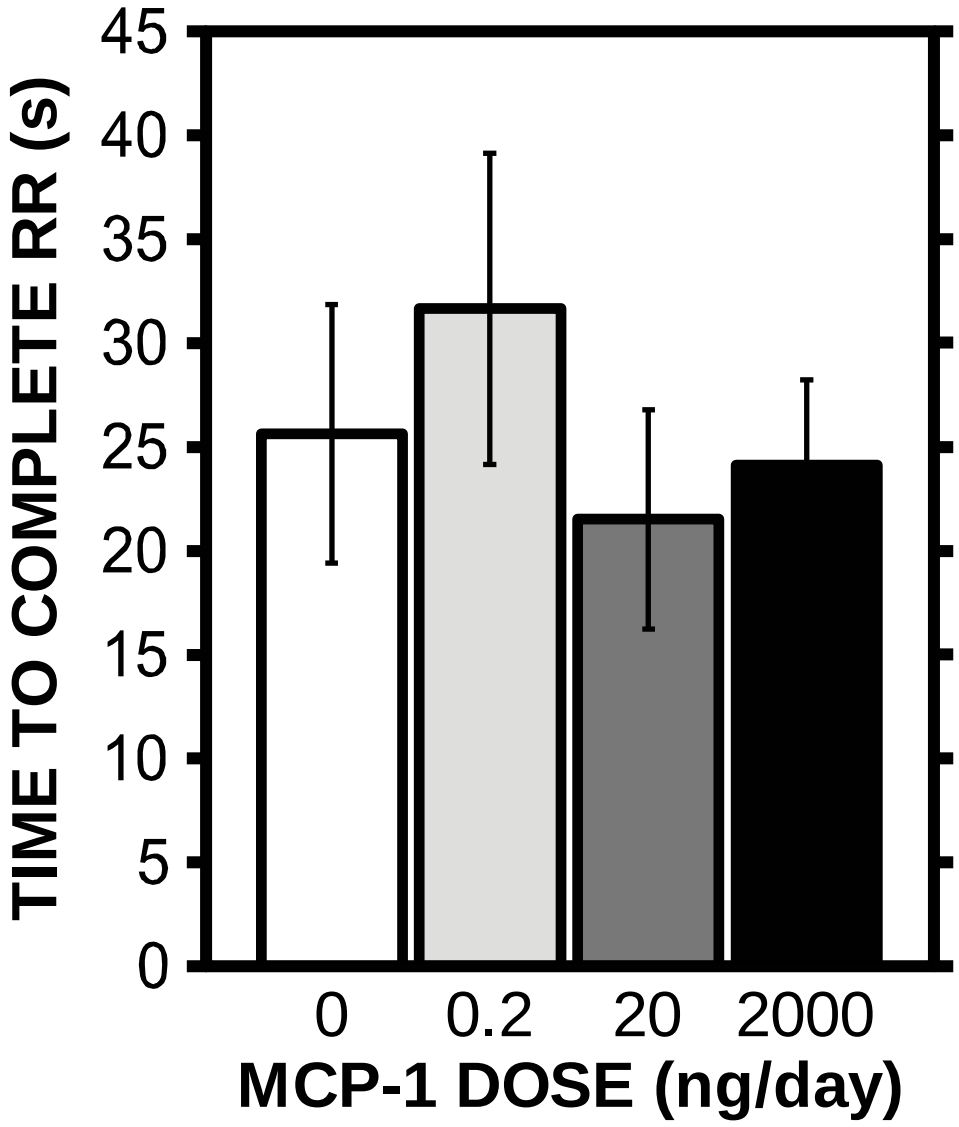

Figure 2.7: The effect of MCP-1 on the time to reach the response requirement.

There was no effect on the time to reach the response requirement (4 lever presses to gain access to the ethanol solution) across the four weeks of drinking (P=0.88, n=7/6/7/8 for control/0.2/20/2000 ng/day doses respectively). A summary of the data averaged across the first 4 weeks of drinking is shown.

On the final day of pump flow (35 days after pumps were filled, at the end of the 4th week of ethanol self-administration), a progressive ratio test was administered. There was no effect on the consumption of ethanol during a progressive ratio test performed on the last day of pump delivery of MCP-1 (Figure 2.8, $F_{3,23}$=0.33, P=0.80, one-way ANOVA, n=7/5/7/8 for control/0.2/20/2000 ng/day doses respectively). There was no effect on the break point reached during the progressive ratio session (Figure 2.9, $F_{3,23}$=0.39, P=0.76, one-way ANOVA, n=7/5/7/8 for control/0.2/20/2000 ng/day doses respectively). One animal didn't make the progressive ratio analysis because of infection.

MCP-1 did not influence ethanol withdrawal-induced anxiety.

On the final day of pump flow, 6-8 hours after the progressive ratio test was administered, animals were tested for ethanol withdrawal-induced anxiety. During the progressive ratio session, animals had an average intake of 0.83 +/- 0.06 g/kg, which was evenly distributed across doses as indicated by Figure 2.8. During the five-minute anxiety test session, animals were paired with an unfamiliar animal matched by dose. There was no effect of dose on social interaction during the test (Figure 2.10, $F_{3,20}$=1.11, P=0.37, one-way ANOVA, n=6/4/6/8 for control/0.2/20/2000 ng/day doses respectively). Three animals were excluded because they did not have a matched-dose partner at the time of the test. There was no effect of dose on locomotion during the test (Figure 2.11, $F_{3,20}$=0.75, P=0.54, one-way ANOVA, n=6/4/6/8 for control/0.2/20/2000 ng/day doses respectively).

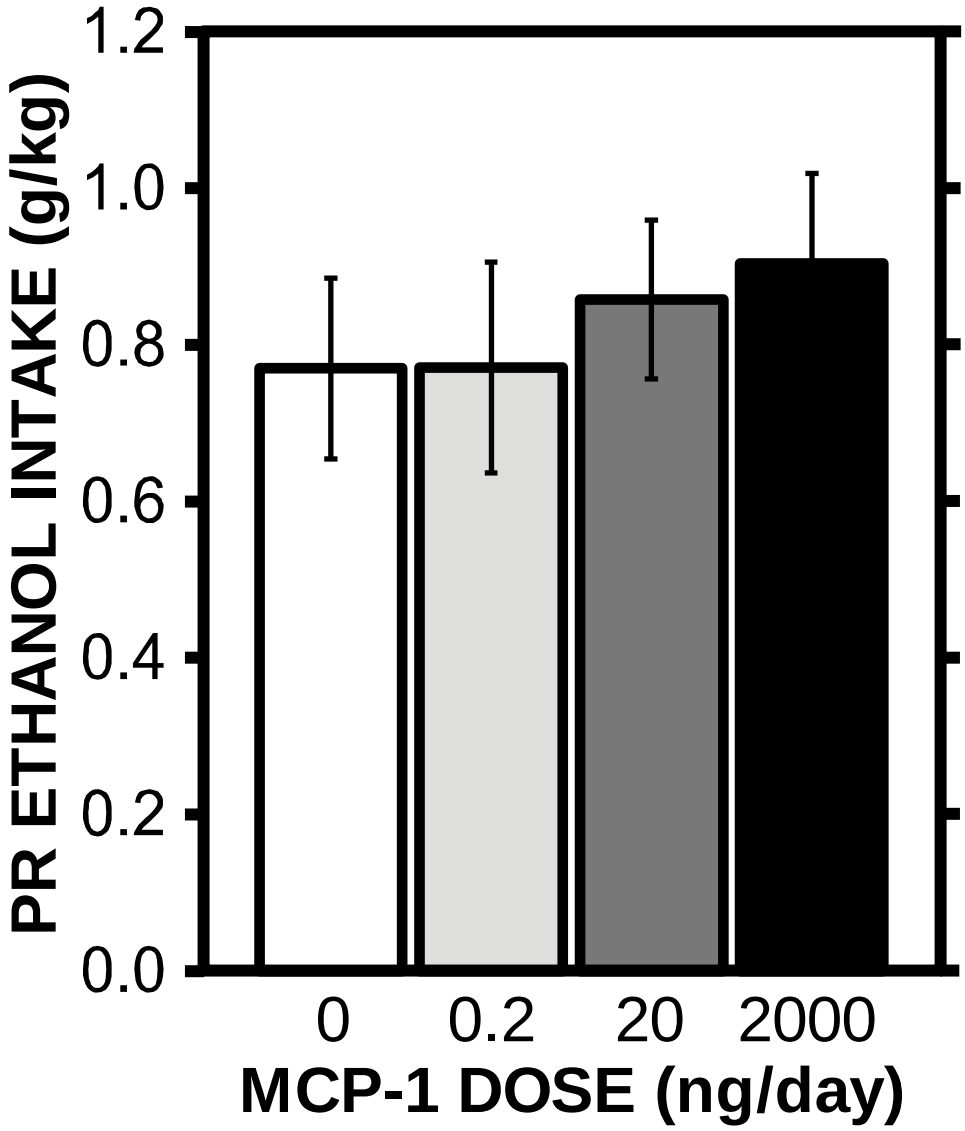

Figure 2.8: The effect of MCP-1 on the ethanol intake during progressive ratio.

There was no effect on the consumption of ethanol during a progressive ratio test performed at the end of the 4th drinking week (P=0.80, n=7/5/7/8 for control/0.2/20/2000 ng/day doses respectively).

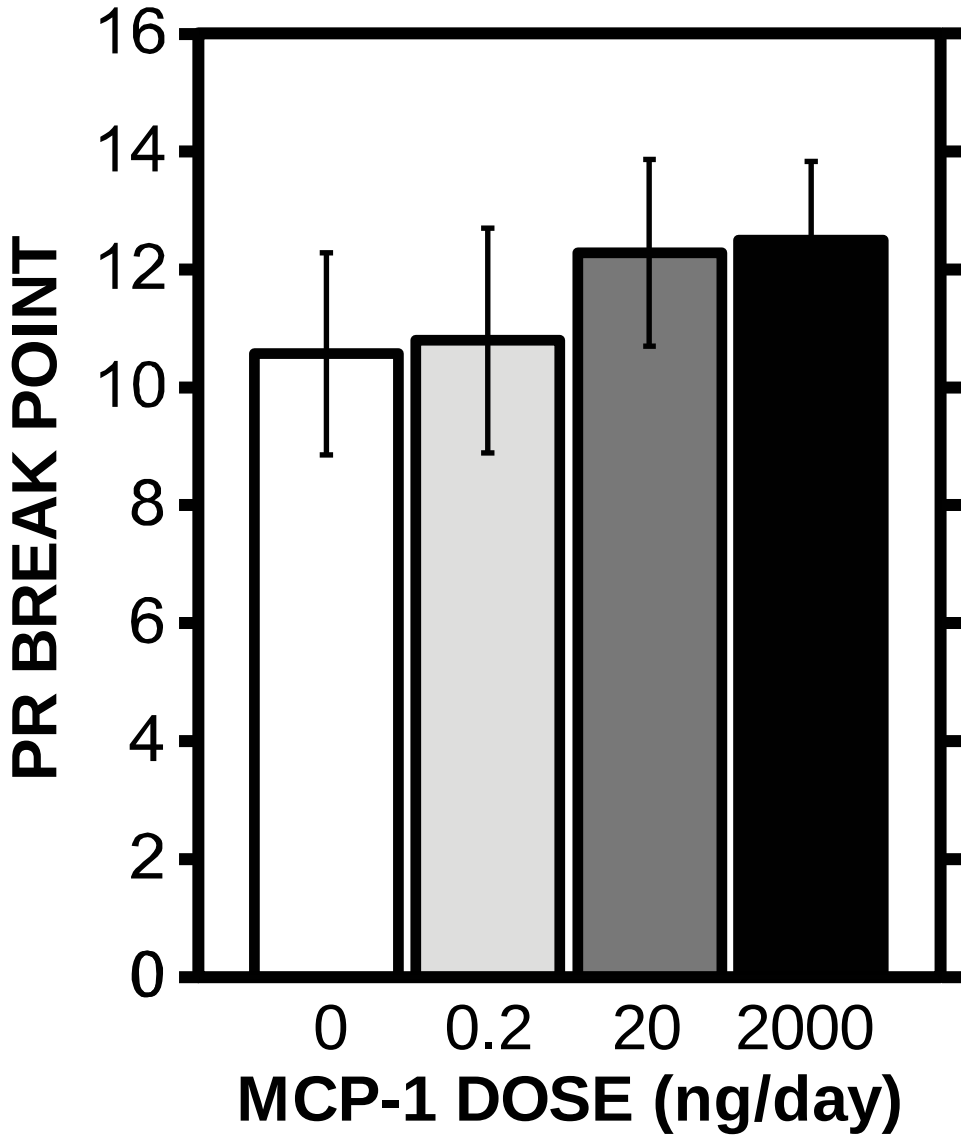

Figure 2.9: The effect of MCP-1 on the break point during progressive ratio.

There was no effect on the break point reached during the progressive ratio session (P=0.76, n=7/5/7/8 for control/0.2/20/2000 ng/day doses respectively).

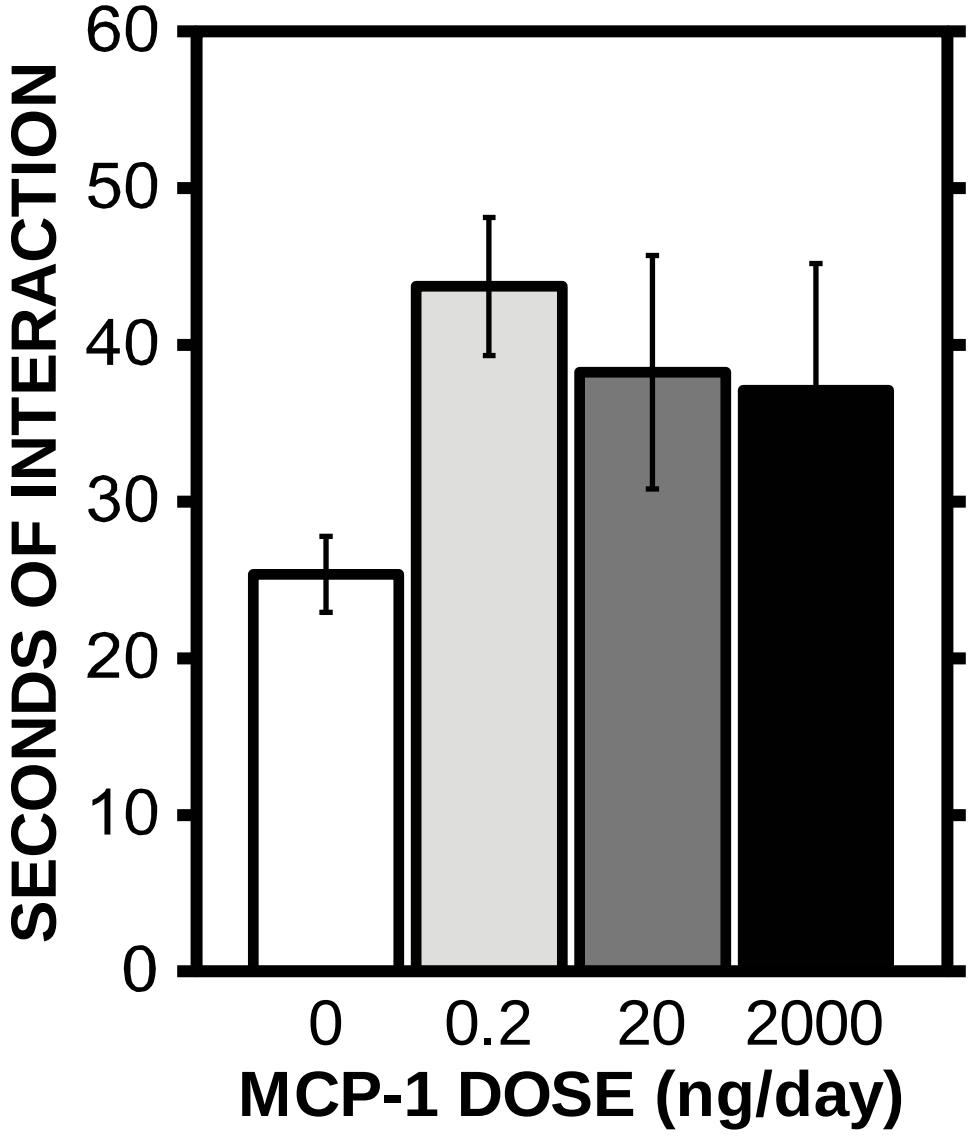

Figure 2.10: The effect of MCP-1 on social interaction during withdrawal.

During a five-minute session, animals were paired with an unfamiliar animal matched by dose. There was no effect of MCP-1 on social interaction during the test (P=0.37, n=6/4/6/8 for control/0.2/20/2000 ng/day doses respectively).

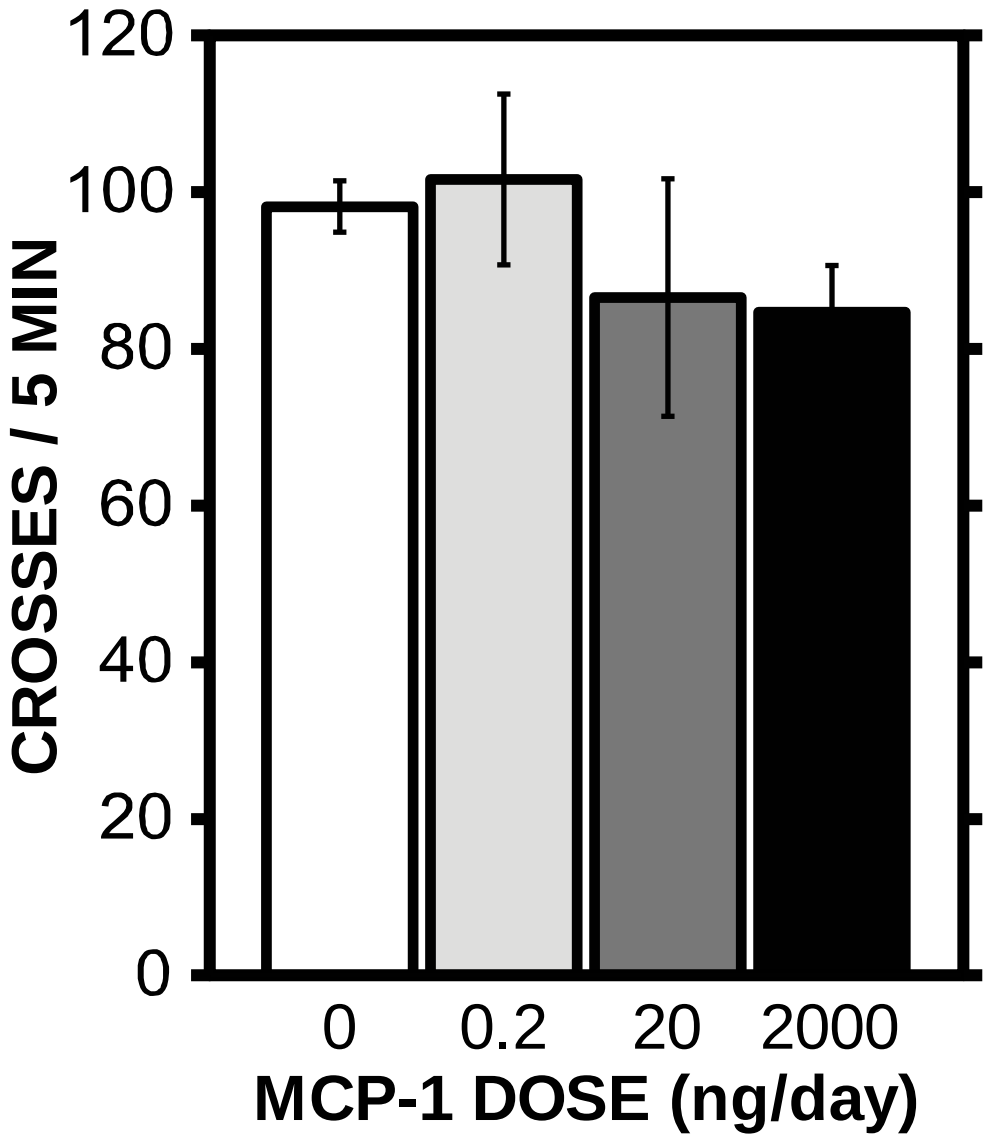

Figure 2.11: The effect of MCP-1 on locomotor activity during withdrawal.

During a five-minute session, animals were paired with an unfamiliar animal matched by dose. There was no effect on locomotion during the test (P=0.54, n=6/4/6/8 for control/0.2/20/2000 ng/day doses respectively).

Stability of MCP-1 during ICV infusion.

ELISA was used to determine the concentration of MCP-1 in the pumps after 14 days (one week after drinking began) and 28 days (3 weeks after drinking began). Pumps were taken from drinking animals that were sacrificed at the day of testing (drinking data not reported because they didn't meet the 4-week criteria for the ANOVA). Data are expressed as a percent of the original concentration of 54 nM (2 ng/day pumps). There were detectable levels of MCP-1 in all pumps tested (Figure 2.12).

Histology

Histological examination confirmed that cannula broke through the corpus callosum into the lateral ventricle in all but one animal, which was discarded from the analyses. Ten placements in the ethanol experiment and two placements in the sucrose experiment penetrated through the ventricle into the fimbria of the hippocampus or the dorsal striatum and were included in the analyses because flow into the ventricle should not have been significantly impacted.

DISCUSSION

Data suggesting that proinflammatory neuroimmune signaling plays a role in unhealthy drinking behaviors has been mounting, but the mechanisms that may contribute

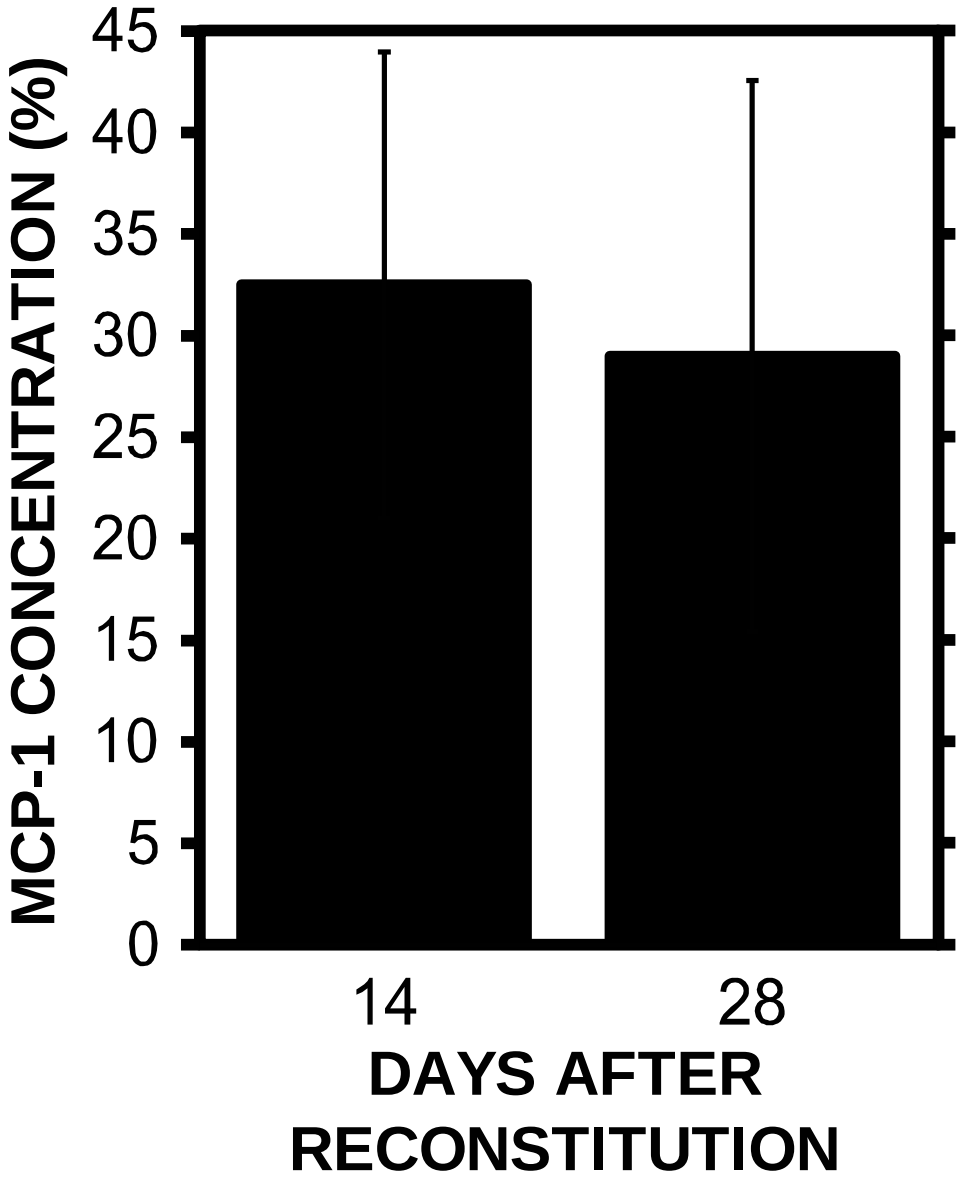

Figure 2.12: The stability of MCP-1 during chronic infusion.

ELISA was used to determine the concentration of MCP-1 in the pumps after 14 days (one week after drinking began) and 28 days (3 weeks after drinking began). Data is expressed as a percent of the original concentration of 54 nM (2 ng/day pumps). N=4 for each time point.

are not completely clear. Our results provide the first evidence of a specific cytokine increasing the self-administration of ethanol. In this experiment, chronic ICV infusion of MCP-1 did not influence the acquisition of sweetened ethanol self-administration (across the first week) but increased consumption across the first 4 weeks (while MCP-1 pumps were flowing) and across the 8-week experiment. The effect of MCP-1 on self-administration developed over several weeks of infusion and several weeks of consumption, with the highest dose of MCP-1 (2 μg/day) yielding the highest consumption during weeks 3 through 8, an effect that persisted for several weeks after MCP-1 delivery ended. These data suggest that increases in MCP-1, whether due to ethanol or various other neuroimmune mechanisms, promotes further ethanol consumption.

Due to a paucity of published data using ICV MCP-1 infusion, particularly chronic infusion, it was necessary for our experiment to test a wide range of MCP-1 concentrations. Since only the highest dose yielded a sustained effect, our experiments may have been limited by the dose or stability of MCP-1. Experiments with higher doses of MCP-1 are necessary to confirm and extend our results, with the possibility that higher doses or increased stability would yield a more robust increase in drinking or a longer-lasting effect.

Chemokines, including MCP-1, participate in a variety of "normal" brain functions in addition to inflammation and pathology (for a review, see Réaux-Le Goazigo et al., 2013). The hallmarks of neuroinflammation are microglial activation, leukocyte infiltration, and blood-brain barrier (BBB) permeability, each with potentially deleterious consequences. MCP-1 by itself does not activate microglia but does regulate microglial chemotaxis, leukocyte infiltration, and BBB permeability (Hinojosa et al., 2011; Gunn et

al., 1997). MCP-1 alters BBB permeability in vivo through direct effects on endothelial CCR2 receptors and subsequent tight junction modification, as well as indirectly through the recruitment of monocytes which can then release additional MCP-1 to alter permeability (Stamatovic et al., 2005; Cushing and Fogelman, 1991; Tieu et al., 2009, Gunn et al., 1997). An acute injection of 1 μg caused a very localized breakdown of the blood-brain barrier in hippocampal tissue (Bell et al., 1996) and a 25 μg ICV bolus injection (but not 5 μg – 20 μg) led to BBB permeability as measured by FITC-albumin leakage and leukocyte infiltration detected throughout the brain (Stamatovic et al., 2005). It is difficult to translate bolus injections quantities to chronic pump infusion rates, but Stamatovic and colleagues (2005) also used chronic ICV administration of 5 μg/h for 3 days or 2.5 μg/hr for 7 days to achieve BBB permeability and leukocyte infiltration to a degree comparable to those seen with the 25 μg bolus, which are 30-fold and 60-fold higher concentrations than we used in our experiment. Thus, it is unlikely that our effect on ethanol consumption was due to neuroinflammatory mechanisms. Although an experiment to determine if using a higher dose of MCP-1 could lead to a stronger effect on ethanol consumption is warranted, measurements of leukocyte infiltration, BBB permeability, microglial density and activation, and brain MCP-1 levels, each at various points in time throughout the study, would help determine if MCP-1 would be facilitating ethanol consumption through normal or inflammatory mechanisms.

We speculate that MCP-1 is having a neuromodulatory effect on the rewarding or aversive properties of ethanol consumption or withdrawal, respectively. One possible "normal" mechanism in line with the concentrations of MCP-1 used in our experiment is

the activation of CCR2 receptors on dopamine neurons. An intracranial injection of 50 ng of MCP-1 into the substantia nigra resulted in elevated dopamine levels in the dorsal striatum for 2 hours (measurements were taken every 20 minutes; Guyon et al., 2009). The same study also showed an increase in dopaminergic activity in slices exposed to 10 nM MCP-1, through modulation of potassium currents. A 50 ng bolus ICV injection also resulted in an increase in phosphorylated tyrosine hydroxylase levels in the VTA 24 hours later, while a CCR2 antagonist attenuated the conditioned place preference for methamphetamine (Wakida et al., 2014). It will be critical to determine if MCP-1 levels reached in our experiment, or through ethanol administration alone, can influence dopaminergic activity. The modest influence of MCP-1 on self-administration in our experiment parallels the modest influence of MCP-1 on dopamine seen in these studies. These parallel effects are consistent with a dopamine link in the mechanism of MCP-1 on sweetened ethanol consumption.

We used sweetened ethanol in our experiment in order to maximize the success rate of ethanol self-administration induction and to provide consistently high levels of self-administration in a minimal amount of time. Minimizing induction time was critical in order to study both the acquisition and maintenance of self-administration during limited access sessions within the time-frame of MCP-1 delivery through the osmotic minipumps. The higher intake achieved with the addition of sucrose to the solution (1.0 g/kg with sucrose versus 0.6 g/kg without sucrose in a 20-minute limited-access session is common) increases the likelihood of central pharmacological effects of ethanol. We have previously detected increased mesolimbic dopamine release within the first few minutes of intake

using this model (Carillo and Gonzales, 2011; Howard et al., 2009), leading us to believe that the BAC range reached (~0.05 % BAC) is reinforcing. Others have shown that low BAC's are anxiolytic and reinforcing as well (for a review, see Koob 2004). Although we acknowledge the presence of sucrose as a complication, the lack of effect on sucrose self-administration leads us to believe that the interaction was primarily driven by ethanol.

Breese et al. (2008) previously did not show an increase in ethanol intake after two acute ICV injections of 100 ng MCP-1 or other cytokines, or various doses of lipopolysaccharide (LPS). The ethanol intake model was 5 days of 4.5% ethanol liquid-only diet, which corresponds to the timing of (and lack of effect during) our acquisition experiment. We are not surprised by the lack of immediate effect in either study, given the protracted nature of both clinical or animal models of ethanol-induced neuroimmune gene expression or neuroinflammation (Qin et al., 2008; He and Crews, 2008; Valles et al., 2004; Pascual et al., 2007; Pascual et al., 2015; Zou and Crews, 2012; Alfonso-Loeches et al., 2010; Erlich et al., 2012) or neuroimmune-induced increases in self-administration (Blednov et al., 2011). However, comparisons of intake between our operant model and the 4.5% ethanol liquid-only diet used by Breese et al. (2008) should be made with caution due to the forced nature of ethanol consumption in the liquid-only model. Further experimentation using chronic dependence models is warranted.

In the same experiment, Breese et al. (2008) showed an increase in withdrawal-induced anxiety after two weekly ICV injections of 100 ng MCP-1 followed by 5 days of a 4.5% ethanol liquid-only diet. Their experiment did not show increased anxiety during withdrawal unless cytokines were administered and previously showed increased anxiety

only after repeated withdrawals (3 cycles) or restraint stress (Breese et al., 2004). They recorded blood ethanol levels at the start of withdrawal from the 5-day ethanol diet between 0.10 – 0.12 % BAC (Breese et al., 2004). The average BAC after our progressive ratio operant session (0.86 g/kg) would be approximately 0.04 % BAC (Carillo and Gonzales, 2011; Howard et al., 2009), which may have been too low to sensitize withdrawal anxiety. Additionally, our animals were dual-housed, in contrast to the single-housed method employed by Breese and colleagues (2004; 2008). However, our animals were paired with an unfamiliar partner and our control animals matched the anxiety levels of the control animals in Breese et al. (2004; 2008). Taken together, the data from our experiment and Breese et al. (2004; 2008) suggest that neither acute nor chronic MCP-1 cause anxiety, and likely exacerbate alcohol withdrawal-induced anxiety only after moderate or greater BAC. The apparent anxiolytic effect of low-dose MCP-1 in our experiment deserves further exploration, particularly in a model of alcohol dependence.

In summary, we discovered that neuroimmune signaling through a specific cytokine can increase the consumption of sweetened ethanol in Long-Evans rats. Our data suggest that ethanol-induced increases in MCP-1, or increases in MCP-1 due to various other neuroimmune mechanisms, may further promote ethanol consumption. Our data add to a growing body of evidence implicating neuroimmune signaling in alcohol use disorders. Continued research into this mechanism, particularly using models of alcohol dependence, will help determine if targeting MCP-1 signaling has therapeutic potential in the treatment of alcohol use disorders.

Chapter 4: Vapor Experiments

ABSTRACT

This chapter will describe my efforts to use ethanol vapor to induce dependence in rodents, with the goal of using dependence to escalate voluntary drinking in a home cage model of self-administration. Vapor inhalation has been shown to increase alcohol drinking in various animal models. I have built ethanol vapor inhalation chambers that could be used to create dependent animals. However, I had trouble obtaining blood alcohol levels within my target range of 0.15 % to 0.25 %, particularly for consecutive days. Changes in ethanol metabolism seemed to occur not only with repeated vapor exposure (in some rats) but also as the rodents aged and gained weight, and these changes were not linear. However, reliable data was obtained with older rats (>325 g) and when using 4-methylpyrazole to block ethanol metabolism by alcohol dehydrogenase (ADH). I have also replicated a common home cage drinking model. Both the vapor inhalation data and the home cage drinking data will be presented in this chapter of the dissertation.

GENERAL INTRODUCTION

The definition of "addiction" isn't universal. Even the DSM criteria used by psychiatrists has gone through several revisions over the years and is still being contested today. For example, the DSM-V, which was published in 2013, eliminated the distinction between alcohol abuse and alcohol dependence and combined them into "alcohol use disorder" with various degrees of severity (2-3 symptoms = mild, 4-5 = moderate, 6+ = severe). However, what's clear is that drug intake does not equate to drug abuse or drug addiction. The brain circuitry and mechanisms influencing casual or intermittent alcohol intake, even binge alcohol intake, are likely very different from those influencing intake during addiction, and medication development is currently primarily concerned with the pathological states of addiction. Although animal models of casual or binge intoxication are useful, models of addiction can provide additional evidence to motivate the large financial investments required for human clinical trials.

"Dependence" can be defined by the physical adaptations that occur with repeated exposures to drugs and "physical dependence" is most often associated with the presence of withdrawal symptoms and/or the development of tolerance. "Addiction", sometimes colloquially referred to as "mental dependence", is associated with compulsive, uncontrollable behaviors despite negative consequences. Rodents will voluntarily drink alcohol daily, with some strains capable of reaching intoxication in a daily "binge" (BAC > 0.08 %) but they do not commonly voluntarily drink alcohol to the extent that is defined

by "abuse", “addiction”, or "dependence", with the exception of those selectively bred for ethanol intake (e.g. “P” rats). Therefore, when modeling addiction or dependence in a rodent, alcohol exposure is generally forced upon the rodent. Further distinction can be made when describing whether dependence is induced when the alcohol is involuntarily forced upon the rodent by the experimenter (e.g. ethanol vapor, etc) or if the rodent is forced to voluntarily consume the drug (e.g. ethanol diet). In this document, the term “dependence” refers specifically to the state of physical dependence after repeated exposure to a drug, to distinguish and emphasize the involuntary nature of the dependent state in forced-exposure animal models. Although the brain circuitry and mechanisms between voluntary and involuntary models are undoubtedly different, the information gained from studies of dependence are extremely valuable. Furthermore, it’s possible that a medication that does not influence a model of binge alcohol intake could have an effect on a model of dependence, and vice versa, so negative results in one model shouldn’t rule out experimenting with the other or considering the medication for clinical trials.

One practical - yet still imperfect - way to study the brain circuitry of dependent animals is to first induce dependence and then pharmacologically manipulate voluntary intake during withdrawal. Even if the experimenter has forced the animal to become dependent on a drug, the animal will still voluntarily consume the drug, particularly in withdrawal, and likely to a greater extent than they otherwise voluntarily would when not dependent. This procedure is commonly used when studying alcohol intake; animals are made dependent through the forced intake of alcohol, and then the voluntary intake of

alcohol during withdrawal is measured and pharmacologically manipulated (for a review, see Vendruscolo and Roberts, 2014).

There is evidence of neuroimmune signaling being enhanced after both acute and chronic ethanol exposure. However, a majority of evidence has been gained from the study of chronic exposure, including clinical or animal models of ethanol-induced neuroimmune gene expression or neuroinflammation (Qin et al., 2008; He and Crews, 2008; Valles et al., 2004; Pascual et al., 2007; Pascual et al., 2015; Zou and Crews, 2012; Alfonso-Loeches et al., 2010; Erlich et al., 2012) or neuroimmune-induced increases in self-administration (Blednov et al., 2011). Therefore, I originally planned to use animal models of dependence in addition to non-dependent ethanol self-administration to research MCP-1 signaling.

Vapor exposure is a preferred method of forcing alcohol exposure for inducing dependence in a rodent. It is a non-invasive procedure that allows precise control over the dose, duration and pattern of exposure. Evidence that chronic ethanol vapor exposure can increase ethanol self-administration in Wistar rats can be found in Gilpin et al. (2008), Gilpin et al. (2009), O'Dell et al. (2004), Richardson et al. (2008), Roberts et al. (1996), Walker and Koob (2007), Funk et al. (2006), and Funk et al. (2007). Our goal was to induce ethanol dependence in Long-Evans rats over 4-8 weeks following the protocol of Funk et al. (2006) and to determine if chronic MCP-1 infusion or chronic CCR-2 antagonist infusion would influence self-administration of ethanol during withdrawal. Two experiments were planned. The first experiment was to test the ability of MCP-1 or the

CCR-2 antagonist infusion to influence the escalation of self-administration and would therefore be administered prior to vapor exposure. The second experiment would test the ability of MCP-1 or MCP-1 antagonist to influence self-administration in already-dependent rodents and would, therefore, begin after the animals were made dependent. The formal goal of the former would be to investigate the importance of cytokine signaling in the development of dependence while the formal goal of the latter would be to provide direct evidence that MCP-1 could be targeted as a potential therapeutic mechanism to treat alcohol use disorders. However, as previously mentioned, experiments with MCP-1 or the CCR2 antagonist in models of dependence have not yet begun.

METHODS

Ethanol dependence will be induced using a well-established model that incorporates chronic exposure to ethanol vapor with operant self-administration. The model we plan to use in particular was developed by the laboratory of George Koob, and the technique details can be found in Gilpin et al. (2008) and Funk et al. (2007). In short, animals spend 24 hours per day in chambers that have continuous air flow and intermittent alcohol flow. Ethanol vapor concentration can be adjusted to reach desired blood alcohol concentrations. Generally, alcohol vapor is cycled on for 14 hours a day and off for 10 hours a day, with a target blood alcohol concentration of 150 to 200 mg% at the end of the exposure session. Evidence that this model is more effective at increasing self-administration than continuous vapor exposure can be found in O'Dell et al. (2004). The

intermittent exposure method also provides a convenient daily withdrawal window to measure self-administration. Vapor exposure normally continues for 6 to 12 weeks. Self-administration sessions will begin after 1 week of vapor exposure and will be performed 6 hours into withdrawal (i.e. 6 hours into a regularly scheduled 10 hour "off" phase) either once or twice per week.

Acrylic chambers measuring 2'x2'x2' were designed by our lab and manufactured by a local company (Regal Plastics, Austin, TX). Two standard rat cages fit in each chamber, and rodents may be single or dual-housed depending on the study design (home cage drinking animals must be single-housed in order to associate liquid lost from a bottle with a specific animal). ¼" PVC tubing is used to pump air and ethanol vapor into the chambers and 1" PVC tubing is used to exhaust from the chambers into the fume hood to ensure human exposure is minimized. Two HK-40L air pumps (Matala, Laguna Hills, CA) are used, one to pump fresh air continuously at 5 L/min into each chamber, while a second is used to pump alcohol vapor at variable rates and is turned on/off using a timer (Illustration 1). A third pump and a second timer were also installed later, which pumps additional air, and is turned on when the ethanol vapor flow is turned off. This keeps the total air/oxygen flow constant and increases clearance of ethanol from the chambers after the ethanol vapor flow is turned off. Ethanol vapor is created by pushing air bubbles through ethanol liquid in a large container. I experimentally determined that using a minimum of four 1-gallon containers and keeping them at least 75% full was required for consistent vapor concentrations for our 5-chamber setup. Each 1-gallon jug was fitted with

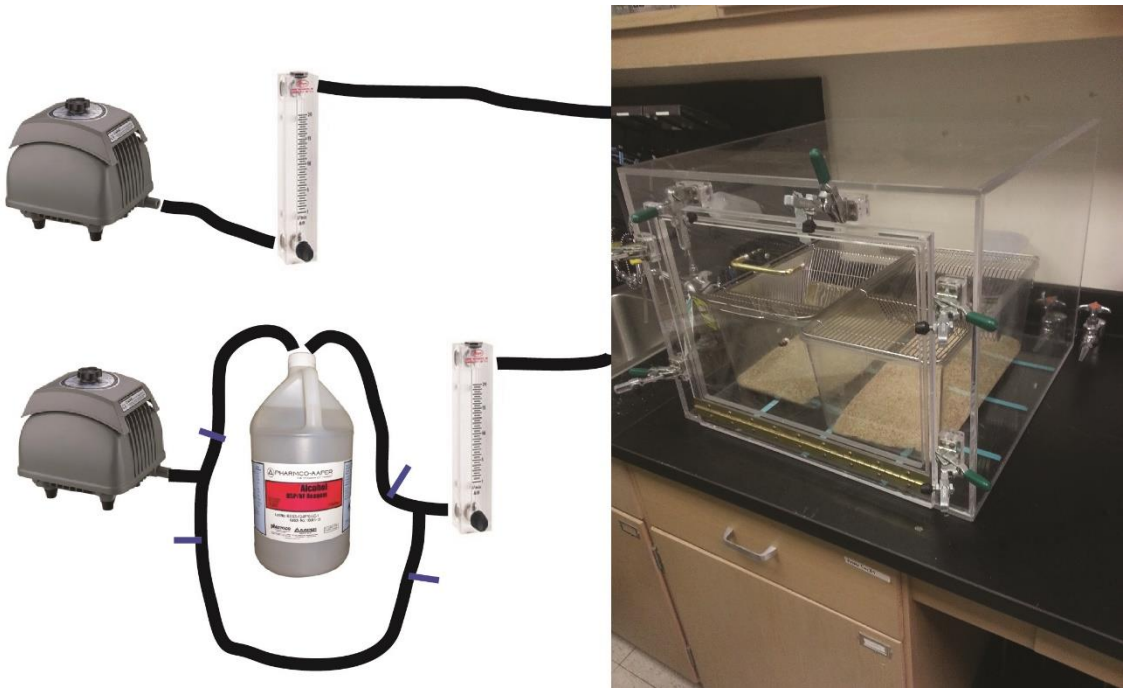

Illustration 1: Vapor chamber setup.

For most experiments, one pump continuously pumped air, while a second pump was switched between air and ethanol. The setup evolved to include a 3rd pump, which was dedicated to air, while the second pump was dedicated to alcohol. Both the 2nd and 3rd pumps were on independent timers, so that when one shut off, the other turned on. This automated the process so that the alcohol could be turned on or off in the middle of the night.

a rubber stopper and a custom cap that would screw over the stopper to keep it from popping off.

Two holes were drilled in the stopper, one for intake and one for exhaust, each with a hard plastic tube pushed through it. The intake tube was connected to a filter/bubbling stone that sat at the bottom of the container with ¼" PVC tubing. The intake was connected to the pump with ¼" tubing, and the exhaust was divided 5 ways to 5 flowmeters. The air pump was also split 5 ways to 5 flowmeters. Flowmeters (Cole-Parmer, Vernon Hills, IL) are used to adjust the flow of air and ethanol vapor individually to each chamber (2 flowmeters per chamber, one for ethanol vapor and one for air). I recommend 10 L/min flowmeters for the air and 5 L/min flowmeters for the ethanol. Generally, 20-35 mg of ethanol per liter of air is used over the 14-hour exposure to reach the desired BAC in our rodents, which has been achieved by using between 2.5 to 4 liters per minute of ethanol vapor while using 5 L/min of direct air. Surprisingly, I have not found any published data on the concentration of ethanol vapor used to achieve specific BAC's in rodents.

Adjusting the flow of air through the ethanol will lead to pre-determined concentrations of vapor, but there is variability among animals due to a number of factors including rates of ethanol metabolism. Therefore, determining BAC's through experimentation is necessary. Once determined, animals will be grouped based on ethanol metabolism rates. We will measure blood alcohol concentrations (BAC) by taking a sample of blood from the saphenous vein and using a gas chromatograph with flame ionization detection to measure ethanol concentrations. In general, ethanol vapor

concentrations are slowly increased between exposure sessions until desired BAC's are reached at the end of session. Other labs (Gilpin et al., 2008; Gass et al., 2014) use a behavioral assessment to gauge rodent intoxication levels, but I found this difficult to do, particularly for my narrow target BAC range of 0.15 to 0.25 % BAC. For example, the method described in Gass et al. (2014) uses the following behavioral guidelines:

100 to 200 mg/dl BAL: rat has a staggering gait

200 to 300 mg/dl BAL: rat has trouble staying on its feet or cannot stand at all

300 to 400 mg/dl (or higher) BAL: rat is totally unresponsive.

I found that dramatic sedation started to occur in animals between 300 and 400 BAL, but only in naïve animals. Tolerance developed unpredictably after repeated exposures, resulting in some animals appearing normal even with 300 BAL. Furthermore, assessing gait was difficult and didn't seem to be a reliable marker of BAC in my rodents. Assessing gait is also difficult while rats are in the chamber, especially during the light cycle when the rodents are generally sleeping. Removing the animals from the chambers to assess gait can also result in the significant loss of ethanol vapor from the chambers when ethanol vapor concentrations need to be adjusted during an exposure session. Therefore, I went through substantial effort to characterize the response of Long-Evans rats to alcohol vapor exposure over time in an attempt to predict their BAC without having to rely on a behavioral measure or to remove the animal from the chamber to take blood samples.

Vapor standards and vapor sampling

Vapor standards were made by injecting known quantities of ethanol liquid (95%, Aaper Alcohol and Chemical Co., Shelbyville, KY) into 1-liter glass bottles with silicone/PTFE septa (the exact volumes were determined by measuring the amount of water necessary to fill the bottle). Up to 100 uL was injected using a gastight microsyringe with a beveled sharp tip, which required up to 30 minutes to evaporate and equilibrate inside the bottle. A 10 ml syringe with a 25 g needle was used to sample 1 mL of air from bottle interior through the septa, and then the syringe was pulled to the maximum volume with room air to dilute to within detectable limits of the GC (the order of this is critical – do not pull air and then ethanol vapor). The 10+ ml of volume in the syringe were then slowly injected into a 2 ml GC sample vial through the septa of a loose cap, overfilling the vial several times over, taking approximately 5 to 10 seconds to complete. The cap was tightened as the injection was nearing completion. Four concentrations spanning the range of possible vapor concentrations in the chambers was used (typically 10/40/70/100 uL) and very consistent results were obtained with typical r^2 values of 0.999.

The vapor chambers were sampled in a similar way. A Luer-lock access port was installed on the front of each chamber which allowed for fast and easy sampling. The same syringe/needle method was used and injected into 2 ml GC sample vials with the same method.

A Bruker 456-GC gas chromatograph with a flame ionization detector and a Varian 8200 headspace autosampler was used to quantify ethanol in vapor and vapor standard preparations as well as in blood and blood standard preparations. A Solid Phase Micro-Extraction (SPME) fiber was used to sample vial headspace with an adsorb time of 0.01 min (0.6 s). The stationary phase was an HP Innowax capillary column (30m × 0.53 mm × 1.0 μm film thickness) and hydrogen was used as the mobile phase. Resulting ethanol peaks were recorded using Compass CDS software from Bruker (Billerica, MA).

Blood standards and blood sampling

Animals were removed from the vapor chambers individually and placed under anesthesia (isoflurane). Depilatory cream was used to remove hair from the upper back legs of the rodents. Either the medial or lateral saphenous vein was poked with a 25 g needle, resulting in a pool of blood on the skin. 10 uL of blood was pipetted from the pool into a 2 ml GC vial that was prefilled with 90 uL of saturated saline and capped within a standardized amount of time. All blood samples were taken in triplicate. The first session, which required depilatory cream, took approximate 6 minutes per animal, while future sessions only required 3 minutes per animal, minimizing the differences in vapor exposure times. When more than 5 animals were used, their entry into ethanol vapor was staggered, if possible (when not using the timer to begin vapor exposure). External standards were made using 10 uL of known ethanol concentrations pipetted into GC vials with 90 uL of saturated saline.

4-methylpyrazole

Some vapor experiments used 4-methylpyrazole (4MP; Sigma-Aldrich, St. Louis, MO) to block alcohol dehydrogenase (ADH). Greater than 90% of ethanol metabolism is due to ADH, and about 75% inhibition of ADH is achieved with 1mmol/kg pyrazole (up to about 85% with 2mmol/kg) and about 89% inhibition with 1mmol/kg 4MP (Plapp et al., 2015). Not only does pyrazole and pyrazole derivatives like 4MP inhibit ADH, but alcohol also inhibits the metabolism of pyrazole. 4MP has a half-life of approximately 11 hours for 1 mmol/kg, but that turns to approximately 33 hours when administered with a 30mmole/kg dose of ethanol IP (~1.5 g/kg; Blomstrand and Ostling, 1977). In order to determine a starting point for a 4MP dose that could stabilize BAC for a 14-hour vapor exposure session, I used experimental evidence that a 0.88 mmol (60 mg/kg) dose of pyrazole was required to make a 1.5 g/kg dose of ethanol last 15 hours in a rat (Goldberg and Rydberg, 1969), while a dose of 1mmol/kg of 4MP would make that dose last 36 hours (Plapp et al., 1984).

Doses of pyrazole above 80mg/kg, and up to 600 mg/kg have been shown to cause sedation and CNS depression beyond what is normally expected from alcohol (Rydberg, 1969; Ferko and Babyock, 1976). Derivatives of pyrazole such as 4MP have become more common because they are more potent, more specific, and less toxic than pyrazole (Deis and Lester, 1979; Ferko and Babyock, 1976). We chose to use 4MP because of known pharmacokinetic data, increased potency and selectivity over pyrazole, less toxicity than

pyrazole, and were partial to 4MP because our lab has published data using 4MP in Long-Evans rats in the past.

Ethanol Metabolic Rate

Ethanol metabolism rates were determined by taking consecutive blood alcohol measurements for several hours. The animal was removed from animal vapor, blood was drawn, and the animal was placed in fresh bedding outside of the vapor chambers. If the animal's BAC was greater than zero, 1 hour later blood was drawn again. The process was repeated until an animal's BAC reached 0, or 3 hours passed (4 points), whichever occurred first. Generally speaking, animals were taken out of ethanol vapor one at a time, so that each animal's start point was staggered by about 2-3 minutes. Ethanol metabolism rates were determined by best-fit slope for all points above 0 that were collected.

RESULTS

Chamber Testing

My first goal was to show my ability to make standards and to control the concentration of alcohol vapor in the chambers. I turned the air flow to 5 L/min and the

alcohol vapor flow to 0.2 L/min for the first chamber, 0.3 L/min for the second chamber, and so on and measured the vapor concentration at various time points (Figure 3.1).

I realized that the concentration of ethanol may be influenced by presence of rat cages, including food, bedding, and possibly urine, so I determined the effect of having one or two rat cages in a chamber, as well as the effect of having the standard amount of bedding (one scoop) compared to triple the amount of bedding (3 scoops). I used 3 L/min of air and 2 L/min of ethanol vapor for this demonstration. The chamber with only one cage with one scoop filled the fastest, while the chamber with 2 cages, each with 3 scoops, filled the slowest (and thus absorbing more ethanol), and the maximum concentration achieved seemed to be affected likewise, although conclusions cannot be made with just one sample for each condition (Figure 3.2). Interestingly, it wasn't the quantity of bedding that had the biggest initial effect, but rather the surface area of the bedding, as evidenced by the 2 cages / 1 scoop condition filling slower than the 1 cage / 3 scoop condition. However, at the 15-hour time point, the 2 cages / 1 scoop condition seemed to catch up and eventually surpassed the 1 cage / 3 scoop condition.

Similarly, the emptying of ethanol vapor from the chambers (by leaving the air flow on but shutting off the ethanol vapor flow) was influenced by the presence of bedding in the same way (Figure 3.3). Again, surface area was key, with the 1-cage conditions clearing the fastest and the 2-cage conditions clearing the slowest.

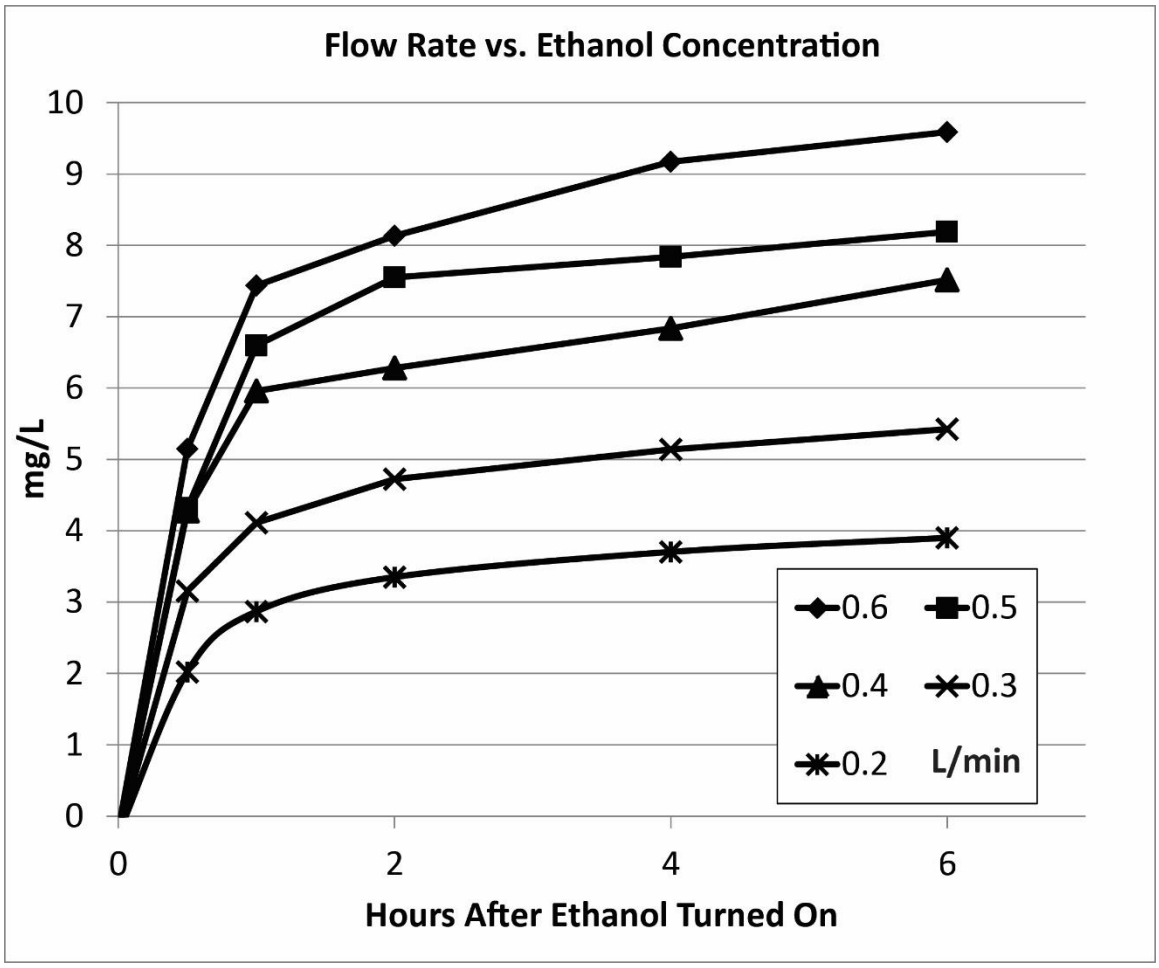

Figure 3.1: The control of ethanol vapor concentration in the vapor chambers.

Using 5 L/min of air and various flow rates of ethanol, I sampled the chambers at various points in time.

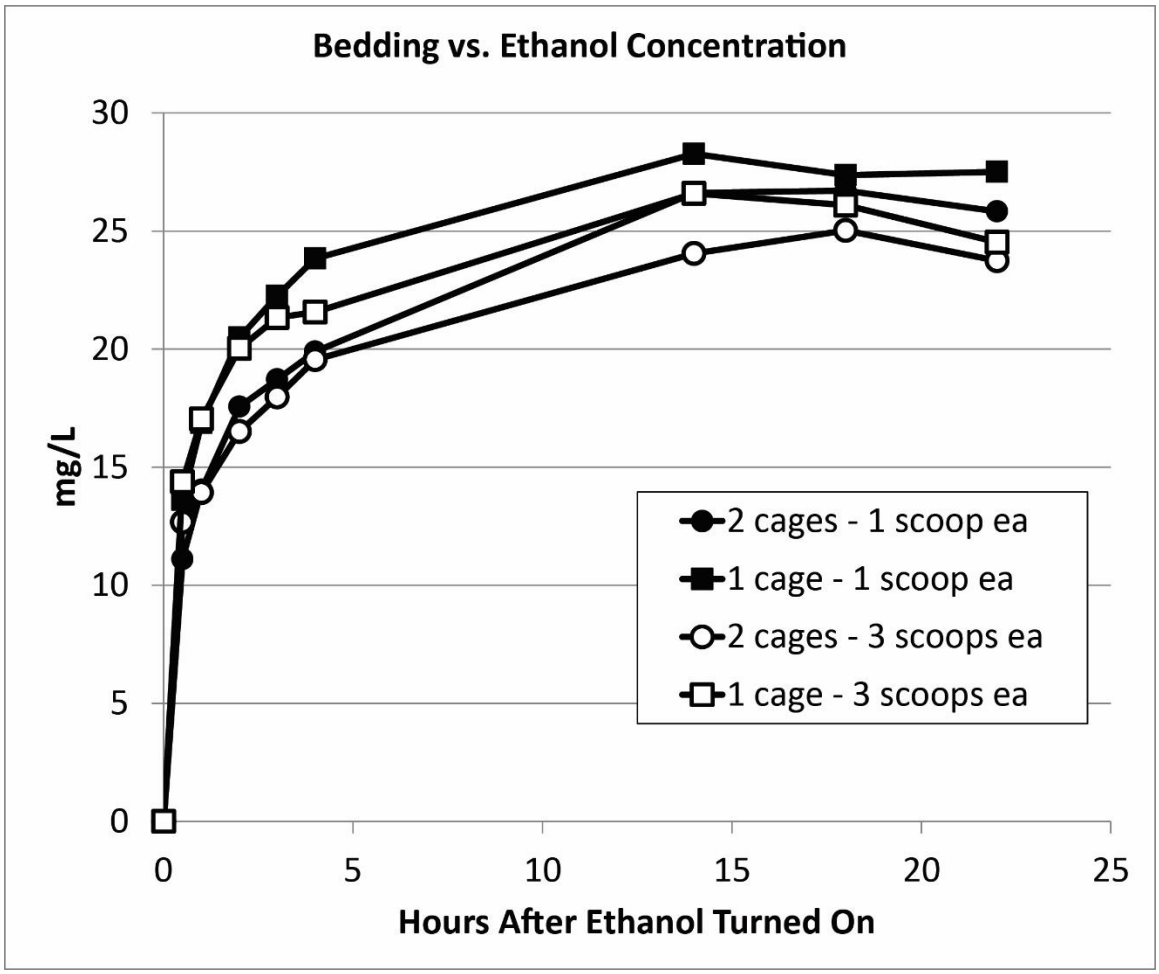

Figure 3.2: The effect of bedding on ethanol concentration.

The chamber with only one cage with one scoop filled the fastest, while the chamber with 2 cages, each with 3 scoops, filled the slowest, and the maximum concentration achieved seemed to be affected likewise, although conclusions cannot be made with just one sample for each condition.

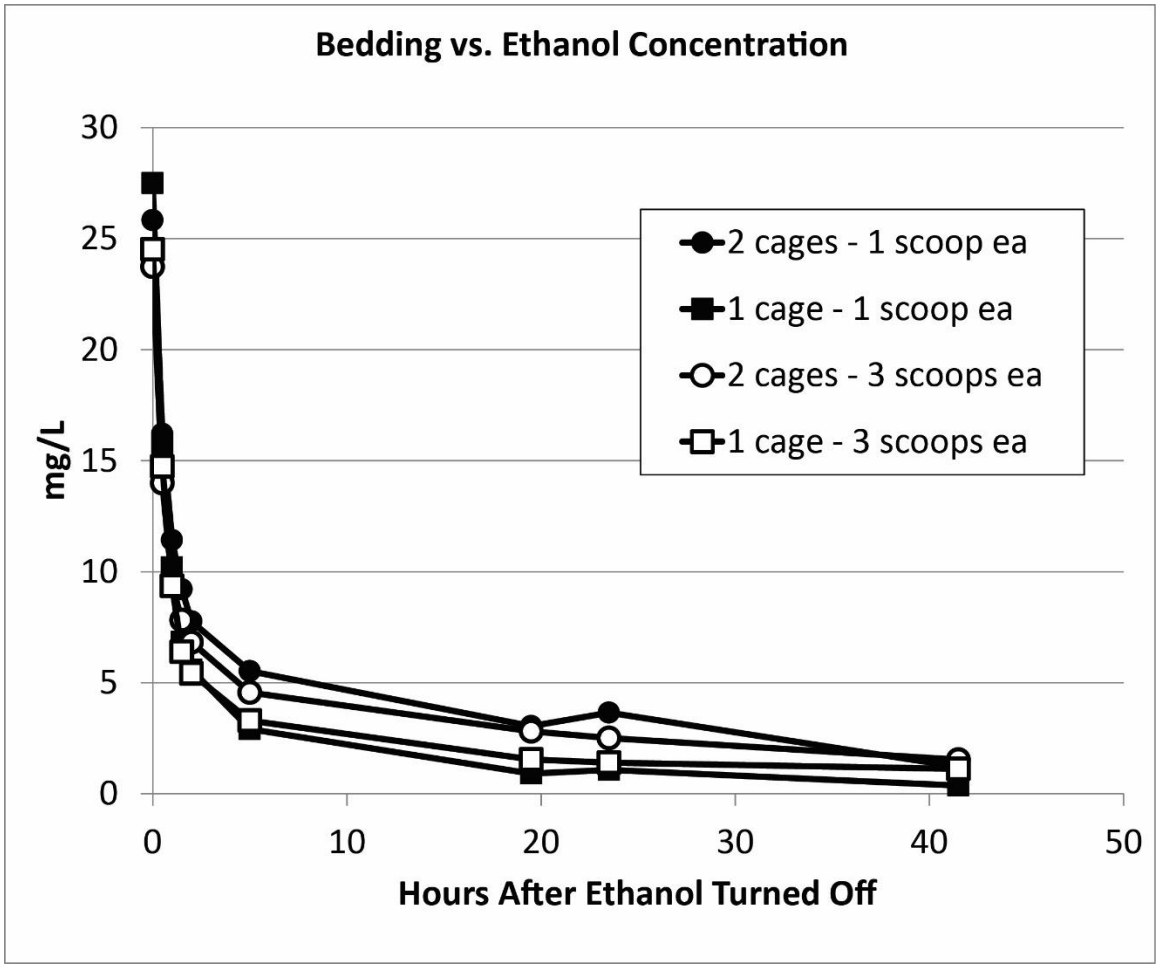

Figure 3.3: The effect of bedding on ethanol clearance.

Surface area was key, with the 1-cage conditions clearing the fastest and the 2-cage conditions clearing the slowest.

Rodent BAC

Because of the lack of data regarding ethanol vapor concentration and resulting BAC's, my first experiment with rodents was to measure BAC after only brief exposures. Using 2.5 L/min of air and 2.5 L/min of ethanol vapor, I pre-filled the chambers overnight, leading to chamber ethanol vapor concentrations of 30-35 mg/L. I measured the BAC of 5 rodents, weighing an average of 442 ± 14 grams after 2 hours, then again after 4 hours and 6 hours (Figure 3.4 A/B). 5 days later, I repeated the experiment, but with blood draws taken after 8, 10, and 12 hours. Finally, I repeated it a third time 5 days after that, with bloods drawn after 14, 18, and 22 hours. Some animals showed a clear ethanol metabolic tolerance developing by the 3rd experiment, and this was evident in their behavior. Generally, only rats above 0.3% BAC showed a staggering gait, and none of the rats became unconscious during these experiments.

The next step was to expose animals to consecutive days of ethanol vapor. I lowered the ethanol vapor concentration to 25-30 mg/L (2 L/min ethanol vapor and 3 L/min air) and exposed a group of 10 animals (average 464 ± 13 g) to a 14-hour exposure for two consecutive days, with a 10 hour "off" period. However, after the second day the blood alcohol concentrations were very high (range 0.19 to 0.46 % BAC, average of 0.32 %), so I measured the BAC in the rodents after the 10-hour off period to see if the BAC was being cleared prior to the start of the next session. Knowing that the ethanol clearance from the chambers was fast but incomplete, I anticipated that the animal's BAC would drop more

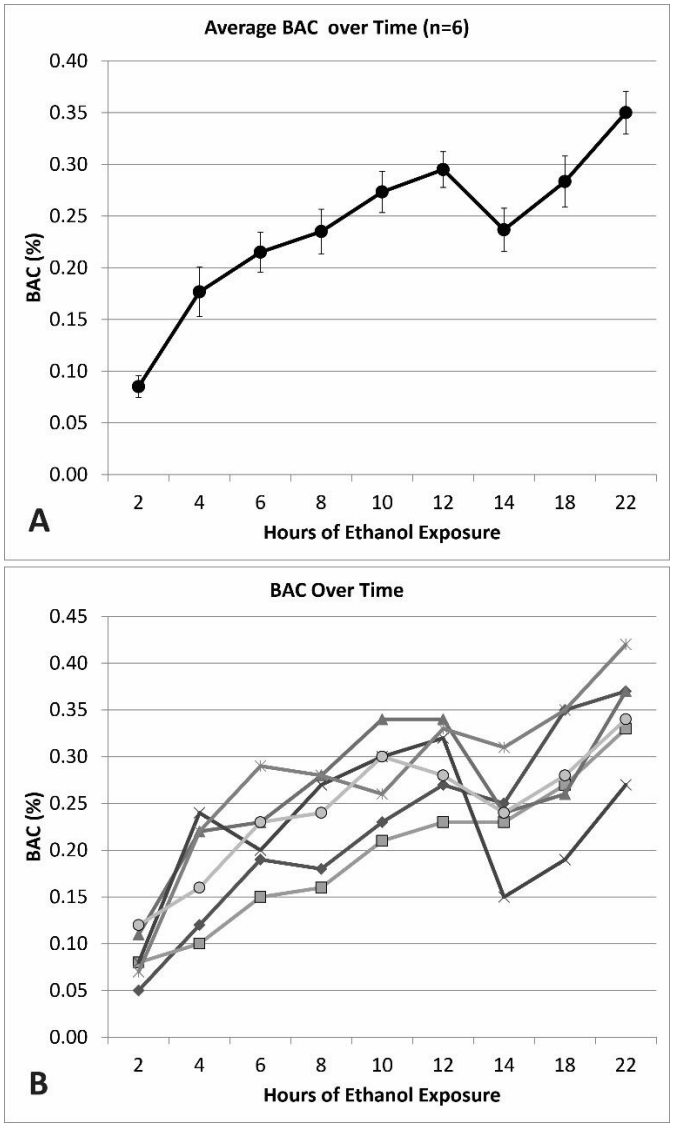

Figure 3.4: Blood alcohol concentration over time.

Rats weight an average of 442 grams were exposed to 30-35 mg/L alcohol vapor for increasing amounts of time. The 2/4/6 hour time points were taken on day 1, the 8/10/12 time points were taken 5 days later, and the 14/18/22 time points were taken 5 days after that. Some animals showed a clear ethanol metabolic tolerance developing by the 3rd experiment. Panel A shows the average and SEM, panel B shows the individual animals.

slowly than if they were removed from the chambers altogether. However, I did not anticipate the BAC to drop from 0.32 ± 0.03 % BAC to only 0.21 ± 0.03 % BAC on average. The change in BAC for the individual rodents is shown in Figure 3.5. Clearly, before proceeding, I had to re-evaluate the clearance of ethanol from the vapor chambers.

I purchased new flowmeters to increase the maximum air and ethanol flow to the chambers while still keeping the ratio the same. I once again determined the fill rate and empty rate of the chambers in the presence of 2 cages with 1 scoop of bedding. I turned the air flow to 5 L/min and the ethanol vapor flow to 3.33 L/min. The maximum ethanol vapor concentration reached was predictable based on the ratio of air to ethanol vapor, but the increased flow rate meant the ethanol vapor concentration increased slightly quicker (data not shown). Also, the clearance of ethanol vapor was much faster, leading to 2-3 mg /L after 4 hours, while previously having just over 5 mg/L after 5 hours at 3 L/min air flow (Figure 3.6). Previous data from a 3 L/min experiment is shown for comparison.

Not being satisfied with this ethanol clearance rate, I modified the system so that the ethanol flow was turned into additional air flow rather than simply being shut off after 14 hours. This could be accomplished by purchasing a 3rd pump and a second timer that would turn on when the 1st timer controlling the ethanol vapor flow shuts off. However, while I waited for that additional equipment to arrive, I simply installed a shunt to manually divert the flow from the ethanol containers directly to the chambers. Due to decreased

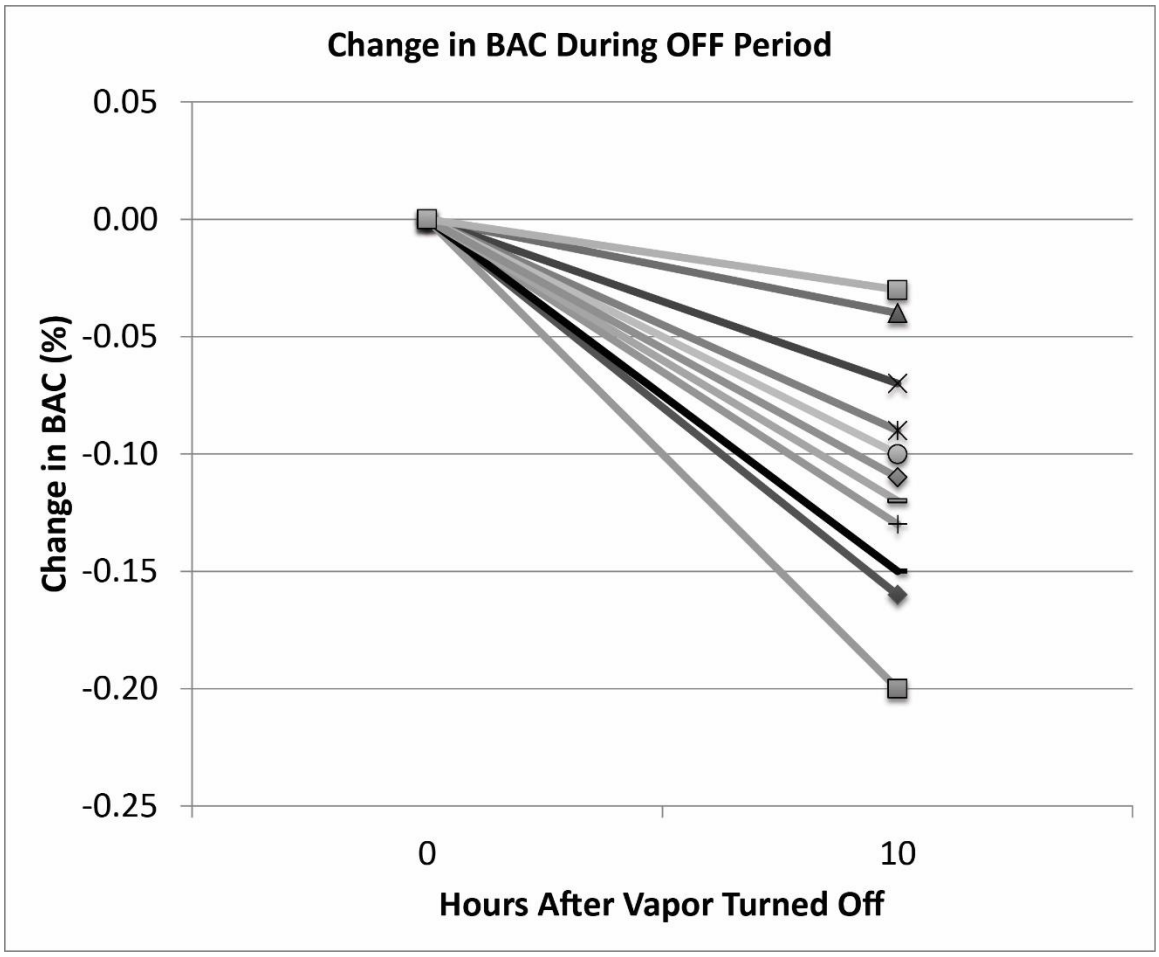

Figure 3.5: The change in BAC for individual animals after ethanol vapor is turned off.

After turning the vapor concentration down to 20-25 mg/L and exposing 10 rats to two 14-hour exposure sessions (with a 10-hour break between), the rodent BACs were very high (range 0.19 to 0.46 % BAC). I measured their BAC 10 hours after ethanol vapor was shut off, but while the rodents were still in the chambers. The change in BAC 10 hours later was surprisingly low (average of -0.11 ± 0.02 % BAC).

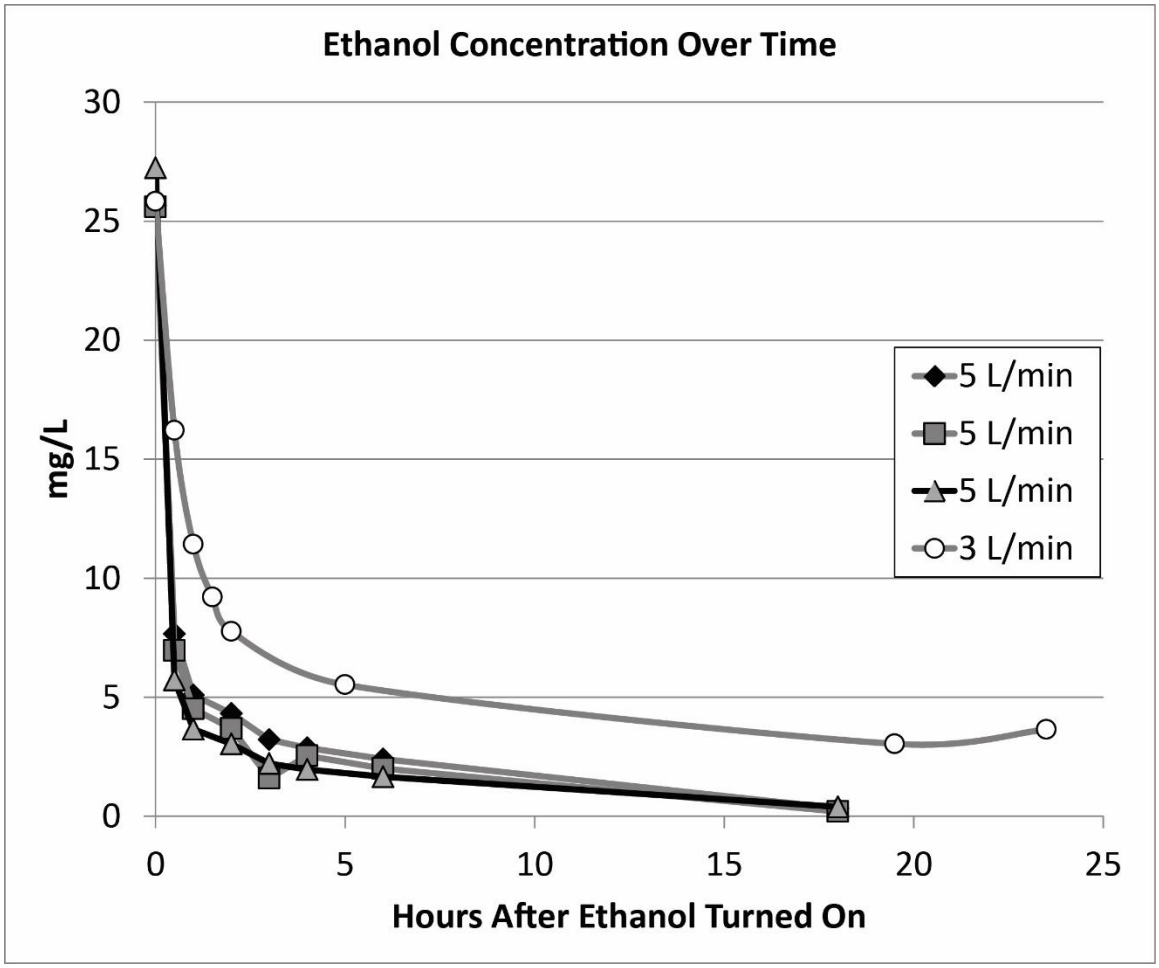

Figure 3.6: The clearance of ethanol vapor at various air flow rates.

I turned the air flow to 5 L/min and the ethanol vapor flow to 3.33 L/min. The maximum ethanol vapor concentration reached was predictable based on the ratio of air to ethanol vapor, but the increased flow rate meant the ethanol vapor concentration increased and decreased faster. This graph shows the clearance of ethanol after the ethanol vapor flow is turned off.

resistance, 3.33 L/min ethanol vapor translated to approximately 4.5 L/min air flow, for a total of 9 L/min air flow, (previously 3 L/min before the 2nd pump was installed).

Consecutive Vapor Exposures

Given the variability in ethanol metabolism rates, it became clear that individual chamber vapor concentrations would have to be adjusted based on individual rodent ethanol metabolism rates. Since rodents can be pair-housed and each chamber can fit 2 cages, adjusting individual chambers and swapping animals between chambers can get complicated. I attempted to use consecutive 14-hour exposures to bring 10 rats to within the ideal range of 0.15 to 0.20, with a range of 0.15 to 0.25 also being acceptable. I used 510 ± 13 g rats that had been drinking an average of 0.6 g/kg daily (over 1 hour) in the home cage for at least one month prior (but did not drink on days they were exposed to ethanol vapor). The rats were exposed to three consecutive days of vapor and then had the weekend off (but drank in the home cage) before resuming ethanol vapor exposure for three more days (Figure 3.7). I modified ethanol flow rates between exposure sessions based on BAC after the previous exposure session and did not changes flow rates during an exposure session. Flow meters were set at 5.0 L/min air and between 3.0 – 5.0 L/min ethanol, resulting in ethanol vapor concentrations of 23.2 – 31.5 mg/L. I was unable to maintain BAC's within an acceptable range during those days. However, I confirmed that most animals were easily clearing all of their blood ethanol within the 10-hour off periods, even with initial BAC's as high as 0.35 % (data not shown). Not surprisingly, however,

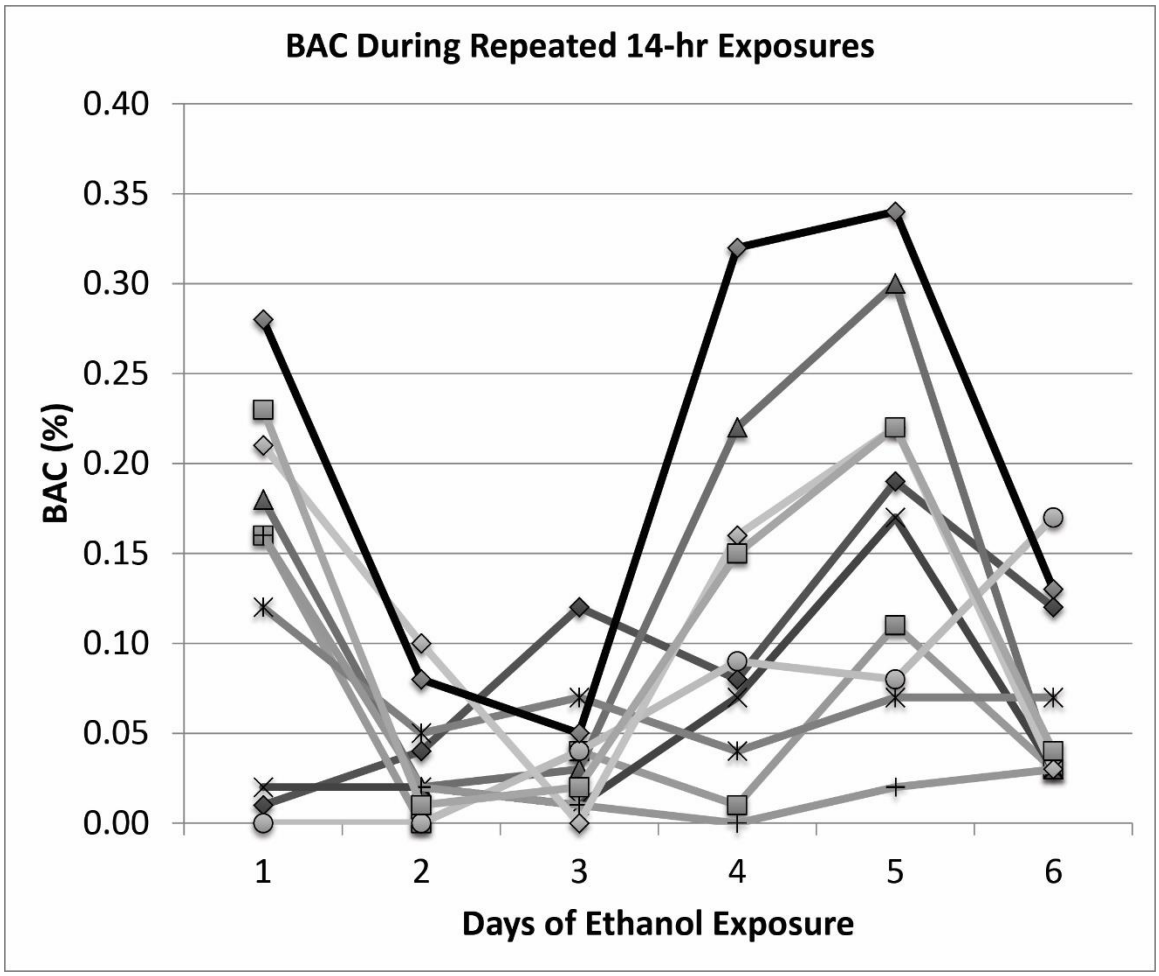

Figure 3.7: BAC after consecutive days of ethanol vapor exposure.

440 g rats that had been drinking an average of 0.6 g/kg daily (over 1 hour) in the home cage were exposed to three consecutive days and then had the weekend off (but drank in the home cage) before resuming ethanol vapor exposure for three more days. I varied the vapor concentration in the chambers and swapped rats between chambers based on BAC's. Flow meters were set at 5.0 L/min air and between 3.0 – 5.0 L/min ethanol, resulting in ethanol vapor concentrations of 23.2 – 31.5 mg/L. I was unable to maintain BAC's within an acceptable range during those days.

there were three animals that did not clear their blood ethanol after the 4th exposure, even with low initial concentrations (0.15, 0.16, and 0.22), even while their cage mates (who had up to a 0.35 % BAC) had cleared their blood ethanol.

Ethanol Metabolism and Weight

Given the extreme range of BAC's, I performed a series of experiments with the goal of clarifying the factors that were contributing to create such a wide range. I had already alluded to the use of home cage drinkers in the vapor chambers, which was motivated not only by the goal of measuring the influence of vapor exposure on home cage drinking, but also to investigate whether regular alcohol exposure could stabilize ethanol metabolism rates.

The first experiment I performed compared the BAC's of two groups of 5 ethanol-naïve rodents with dramatically different weights (274 ± 4 g and 475 ± 2 g) that were exposed to the same vapor concentrations. There was one animal from each group in each chamber, the air flow was 5 L/min and the ethanol vapor flow was 3.33 L/min, resulting in all chambers having between 24 and 29 mg/L ethanol. I measured their BAC's after 3, 6, 10, and 14 hours of vapor exposure and then for 3 consecutive hours after being removed from ethanol vapor and put into fresh bedding to determine true ethanol metabolism rates. After 14 hours, the two groups had dramatically different BAC's, with the 275 g group averaging 0.20 % BAC, while the 475 g group averaged 0.02 % BAC (Figure 3.8). There

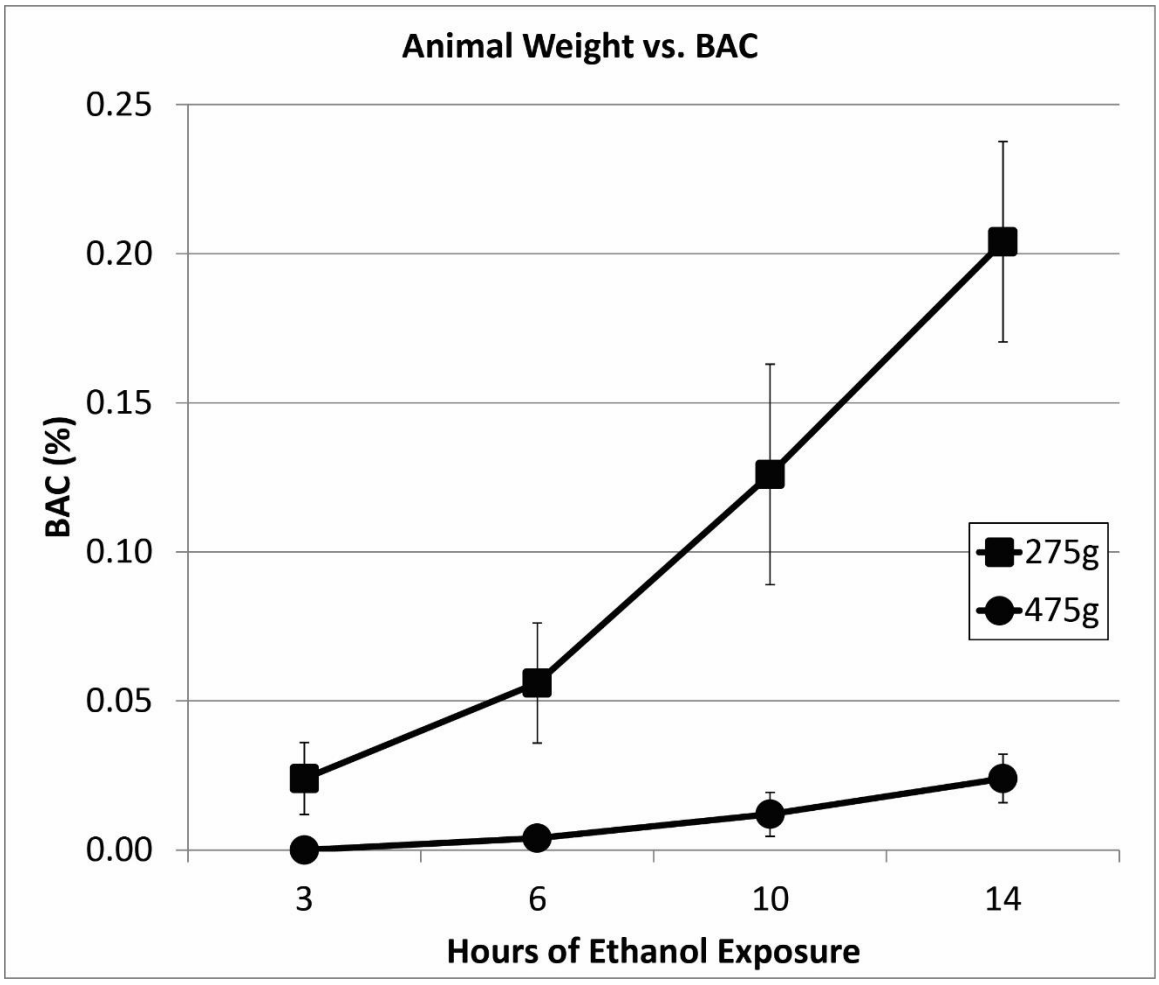

Figure 3.8: The effect of body weight on BAC.

Two groups of 5 ethanol-naïve rodents with dramatically different weights (275 g and 475 g) were exposed to the same vapor concentrations. There was one animal from each group in each chamber, the air flow was 5 L/min and the ethanol vapor flow was 3.33 L/min, resulting in all chambers between 24 and 27 mg/L ethanol). The 275 g group had much higher BAC and much greater variability than the 475 g group. However, there was no difference in ethanol metabolism rates, on average (n=5 per group).

was also substantial variability in the 275 g group but not in the 475 g group. However, there was only a small difference in their ethanol metabolism rates, with the 475 g group having an average rate of -0.047 %/hr (± 0.010 %) while the 275 g group had an average rate of -0.038 %/hr (±0.003). Again, there was a much higher variability in ethanol metabolism rates in the 475 g group than the 275 g group. (Method details - When I recognized that the 475 g group had very low BAC's after the 14-hour exposure, I increased their vapor concentration for several hours to reach an average BAC of 0.07 before measuring their BAC, and then again one hour later, to determine ethanol metabolism rates across 1 hour).

After a 4-day break, I continued to expose the 275 g group for 11 more consecutive days to the same vapor (14 hours on, 10 hours off). Interestingly, their average BAC decreased even after 4 days off, and continued to decrease at a similar rate while being exposed to vapor for the next 11 days (Figure 3.9). On the final day, I compared their BAC's to 5 ethanol-naïve animals from the same cohort (both groups were now about 320 g). Both groups had an average BAC of 0.04.

I measured the ethanol metabolism rates of both the experienced and naïve 320 g groups and re-tested the previous 475 g group, now at 503 ± 5 g (Table 3.1). The repeated exposure group had an increase in their ethanol metabolism rates, from an average of -0.038 %/hr to an average of -0.054 %/hr (± 0.002). Surprisingly, the naïve group had an average ethanol metabolic rate even higher, at -0.064 %/hr (± 0.004). The 503 g group

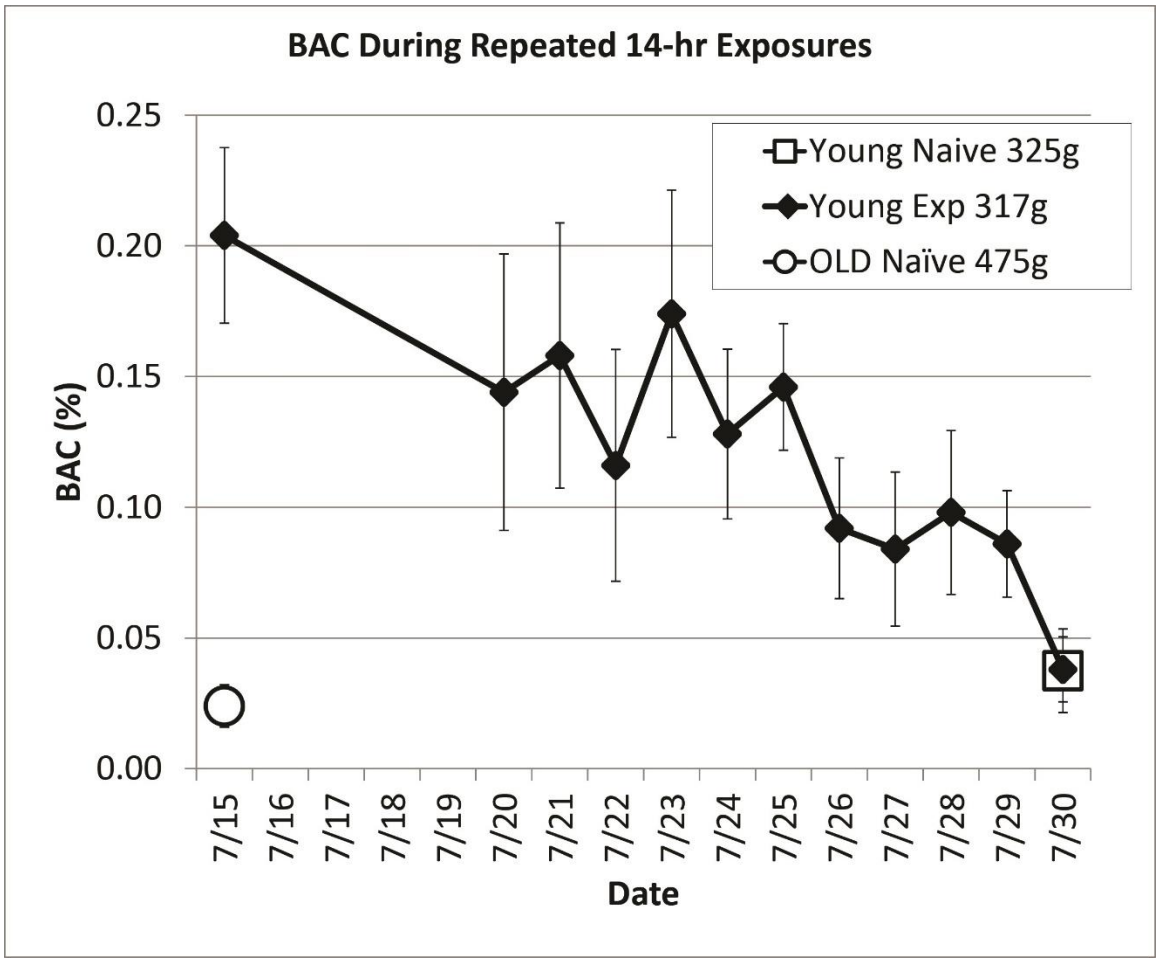

Figure 3.9: The effect of body weight/age and repeated vapor exposure on BAC.

The BAC of rodents declined during repeated exposures but matched those that were not previously exposed. All rats were exposed to the same ethanol vapor (range 24-29 mg/L) for 14 hours per day.

Ethanol Metabolism Rates (BAC per hour ± SEM)		
	7/15/2015	**7/30/2015**
"Old" (n=4-5)	-0.047 ± 0.010	-0.042 ± 0.002
	475 g	503 g
"Young, experienced" (n=5)	-0.038 ± 0.003	-0.054 ± 0.002
(pre/post chronic vapor)	275 g	317 g
"Young, naïve" (n=5)		-0.064 ± 0.004
		325 g

Table 3.1: Ethanol metabolism rates in young and old rats.

Some further insight into the mechanism of BAC decline can be gleaned from comparing ethanol metabolism rates of the rats from Figure 3.9. Although ethanol metabolism rate went up in the "young experienced" group, it was even higher in the "young naïve" group. Ethanol metabolism rate is clearly critical but does not completely explain the differences seen.

stayed within range of their previous measurements, with an average of -0.042 %/hr (± 0.002). The old "post" group (503 g, -0.042, BAC 0.00 is not shown on Figure 3.9 because the ethanol vapor concentrations in their chambers were slightly lower (22 mg/L).

Determining the contribution of body weight to BAC is not a simple experiment. Comparing two groups of different weights but with the similar ethanol metabolism rate can be useful but may also not be very accurate. Although I had three groups with similar ethanol metabolism rates, such as the young "pre" group (275 g, -0.038, BAC 0.21, Figure 3.8) and either the old "post" group (503 g, -0.042, BAC 0.00, data not shown) or the old "pre" group (475 g, -0.047, BAC 0.02, Figure 3.8), I caution against drawing conclusions from this comparison. Further experimentation would be necessary to clarify this interaction for several reasons. First, as previously mentioned, the old "post" group did not have the same ethanol vapor concentration. Second, even minor differences between the groups in ethanol metabolism rates might be significant over such a long vapor exposure. Third, the low n's in each group limits the reliability of the data. Furthermore, several other variables would need to be accounted for and standardized, such as the gas exchange rate in the lungs of the animals. Gas exchange rate is closely tied to both physiological respiration as well as cellular respiration. Specifically, the bulk flow of ethanol into the blood will be dependent on a variety of factors, including respiratory rate, respiratory volume, and BAC. Respiratory rate and volume can be influenced by the size of the animal, its energy expenditure (and thus the time of day), and many other factors. Many of these would be difficult to control.

Given that my goal is to obtain BAC's within a generous range of intoxication (0.15 % to 0.25 %), it may not be necessary to control for gas exchange, time of day, or even the size of the animal. It's likely that simply knowing the ethanol metabolic rates can be enough to accurately predict BAC's after prolonged vapor exposures. A comparison between ethanol metabolic rates and BAC after the 14-hour exposure session is shown in Figure 3.10. A correlation was detected between ethanol metabolism rates vs. BAC's for the five instances (n=24) regardless of age or exposure experience ($r=0.405$, $p=0.049$). A stronger correlation is seen when comparing only across the young animals ($r=0.8256$, $p=0.0002$).

Although ethanol metabolism rate is clearly a factor in determining BAC's obtained during vapor exposure, the data is suggesting that ethanol metabolism rates increase with age or size in adulthood (as opposed to vapor exposure in this case) and may also decline in elderly rats. Only the latter is reflected in the literature. There is a decline in ethanol metabolism rates in elderly rats that is not completely explained by changes in ADH levels or body water content (Collins et al., 1975; Cederbaum, 2012). However, BAC after a given dose of ethanol normally steadily increases as rodents grow from 150 g to 450 g (Bloom et al., 1982). ADH activity also steadily increases with age, but not as quickly as the size of the animal, explaining the increased BAC's reached for a given g/kg dose of ethanol (Bloom et al., 1982). Therefore, the results of my experiments remain unexplained,

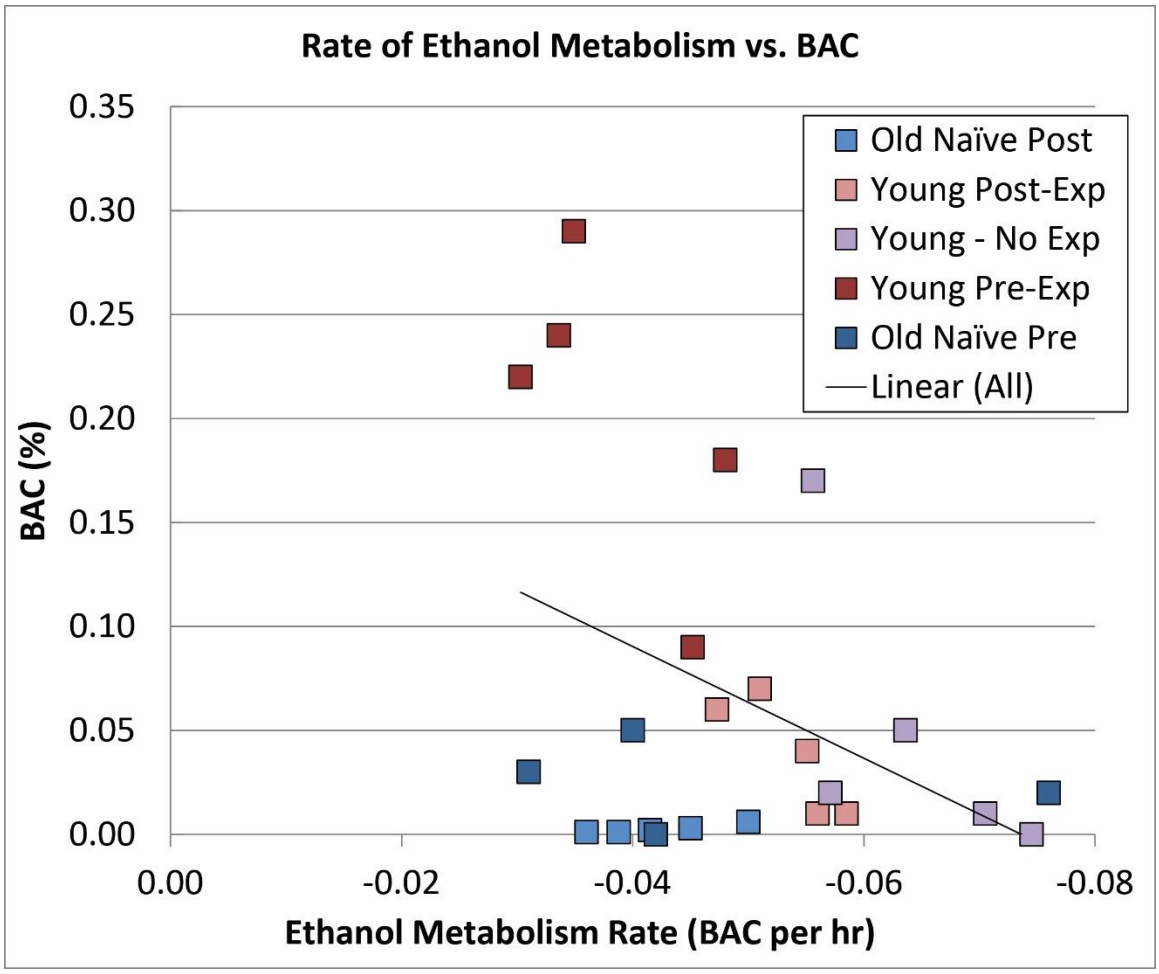

Figure 3.10: Ethanol metabolism rate contributes to BAC in vapor

Statistical analysis of the ethanol metabolism rates compared to BAC's on the five instances (n=24) from Table 1, regardless of age or exposure experience, resulted in a correlation of $r=0.405$, $p<0.05$.

but my expectation is that individual ethanol metabolism rates will explain variations in BAC as long as inhalation rates remain constant. Also, there is no doubt that ethanol exposure can increase ethanol metabolism rates, it's just not evident in this circumstance. A larger effect would undoubtedly be seen if vapor concentrations were escalated to maintain BAC's at the target level. Replications of my experiments with bigger groups of animals would help clarify these details.

Further insight is gleaned from examining the individual changes in BAC of the experienced group while undergoing consecutive exposures sessions. While the group on average declined at a steady pace while in vapor (or not in vapor), the individual rats responded very differently (Figure 3.11). Notice the BAC of some rats increased after the first exposure, while the BAC of some rats increased only after many exposures, and the BAC of others didn't change. Not only is there extreme variability in BAC, there is also extreme variability in the changes in BAC / ethanol metabolism rates induced by exposure to ethanol (or as a result of aging)! Due to the extreme variability in BAC of young rats, and the variability in the changes in ethanol metabolism rate due to exposure, I would not recommend using rats less than 325 grams for vapor experiments.

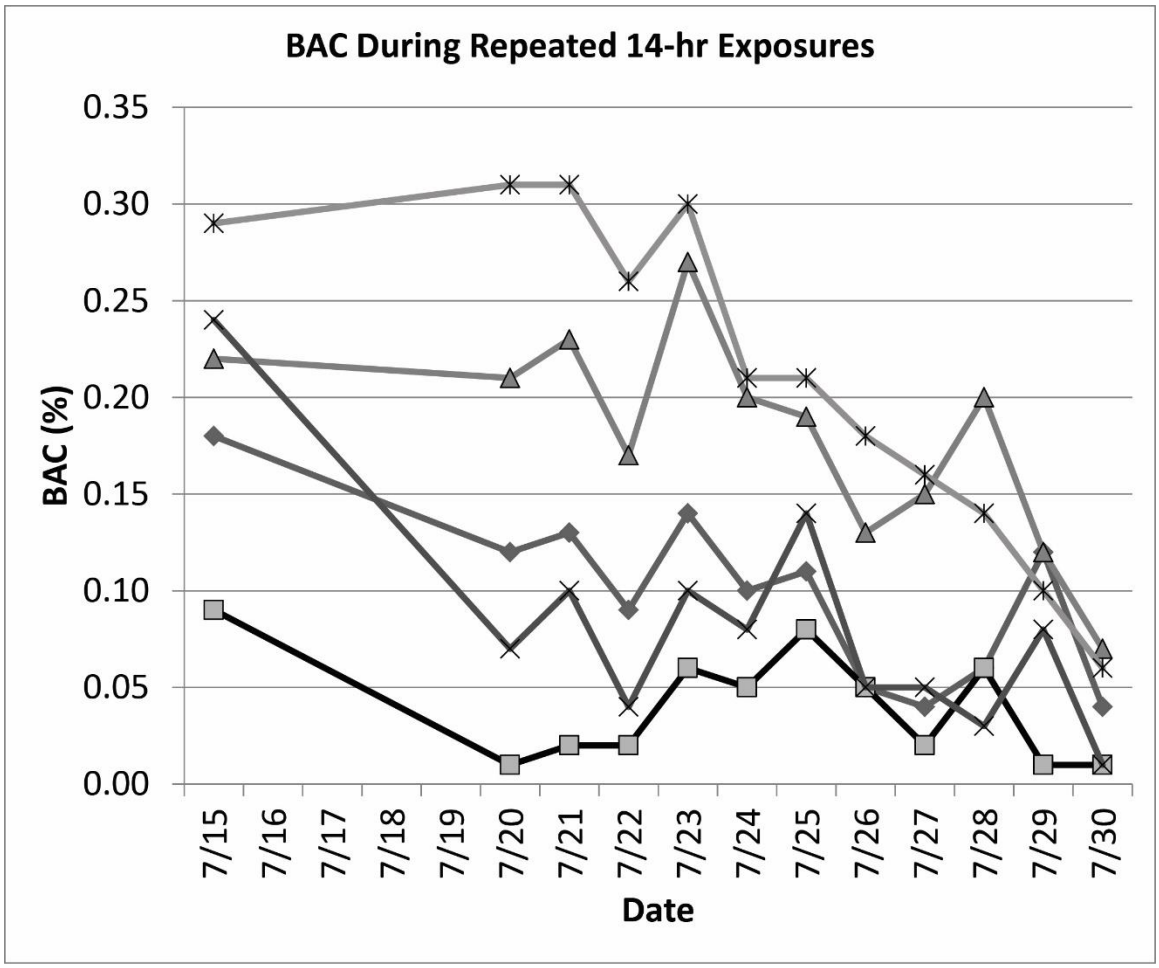

Figure 3.11: Individual BAC's during repeated vapor exposure.

While the group on average declined at a steady pace while in vapor (or not in vapor), the individual rats responded very differently. Notice the ethanol metabolism rates of some rats increased after the first exposure, while the ethanol metabolism rates of some rats increased only after many exposures, and the ethanol metabolism rates of others didn't change. Flowmeters were set to 3.33 L/min ethanol and 5 L/min air, resulting in ethanol vapor concentrations between 24 and 29 mg/L.

Theoretical Model

It's possible that even minor differences in ethanol metabolism rates can lead to dramatic differences in BAC after prolonged vapor exposures. I demonstrate this using a theoretical framework in Figure 3.12, where ethanol vapor is a constant positive input for rats of a given size (and therefore assuming similar inhalation rates), while ethanol metabolism through liver ADH is a constant negative/output. Liver ADH demonstrates 0-order kinetics above 1mM (BAC ~0.005; Cederbaum, 2012). Differences in ethanol metabolism will compound over time (e.g. a difference of 0.01 % BAC/hr will lead to difference of 0.14 % BAC over 14 hours).

Pyrazole Experiments

In order to clarify the contribution of ethanol metabolism rate, I used 4MP to block alcohol dehydrogenase. In the first pilot experiment, my goal was to block ADH maximally. I administered either 10, 41, 82, or 164 mg/kg of 4MP (where 82 mg/kg is 1 mmol), or vehicle, intraperitoneally, to individual rats from the "old" group, now at 590 ± 14 g, one hour before placing them in cautiously-low ethanol vapor concentrations (15-20 mg/L). I measured their bloods after 1 hour, then again after 2, 3, 5, and 7 hours (Figure 3.13). All 4 rats had very similar BAC trajectories, so only the average is shown, with the vehicle animal shown separately with a BAC of 0.00 the entire time. I also measured their bloods after being pulled out of vapor into fresh bedding after 1 hour, then again after 2, 3,

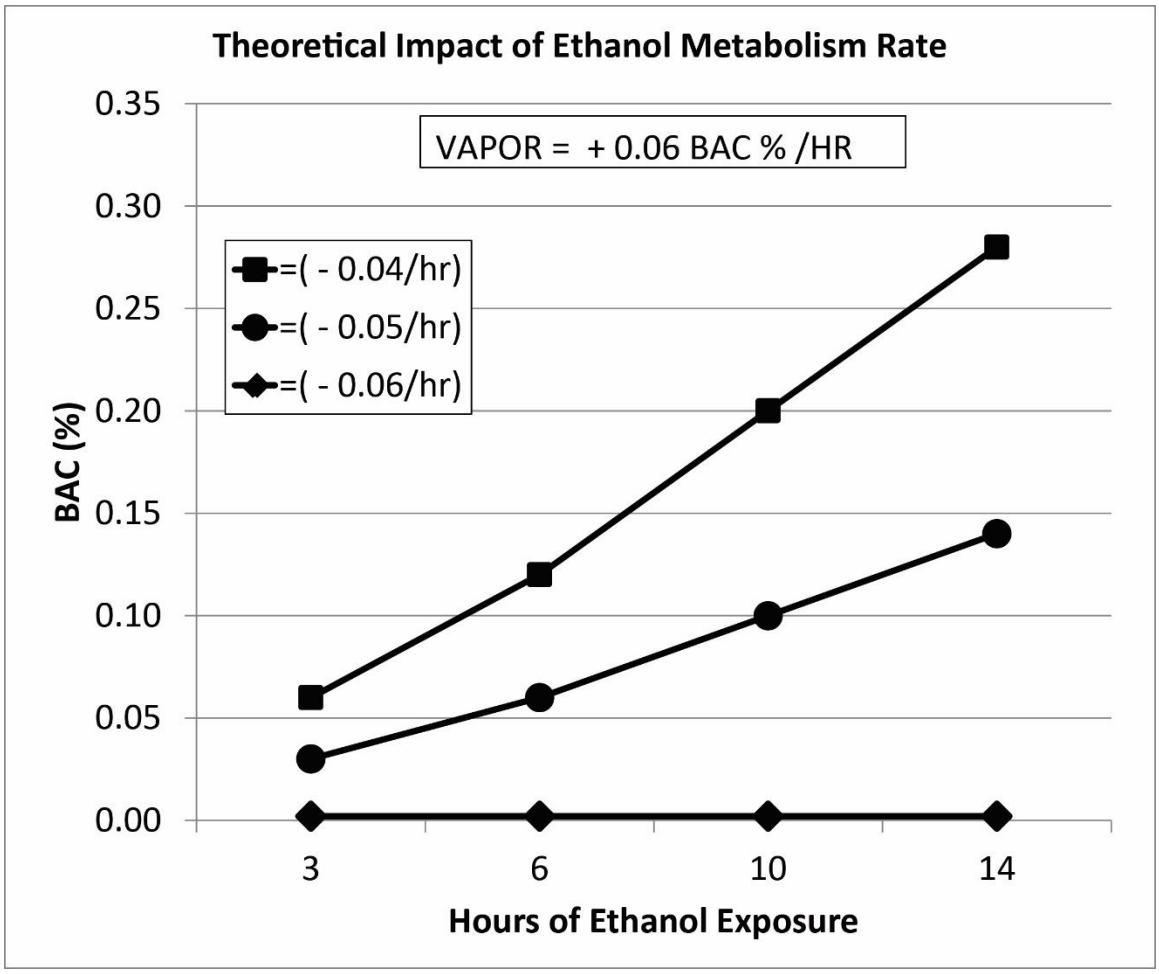

Figure 3.12: Theoretical impact of ethanol metabolism rates to BAC.

It's possible that even minor differences in ethanol metabolism rates can lead to dramatic differences in BAC after prolonged vapor exposures.

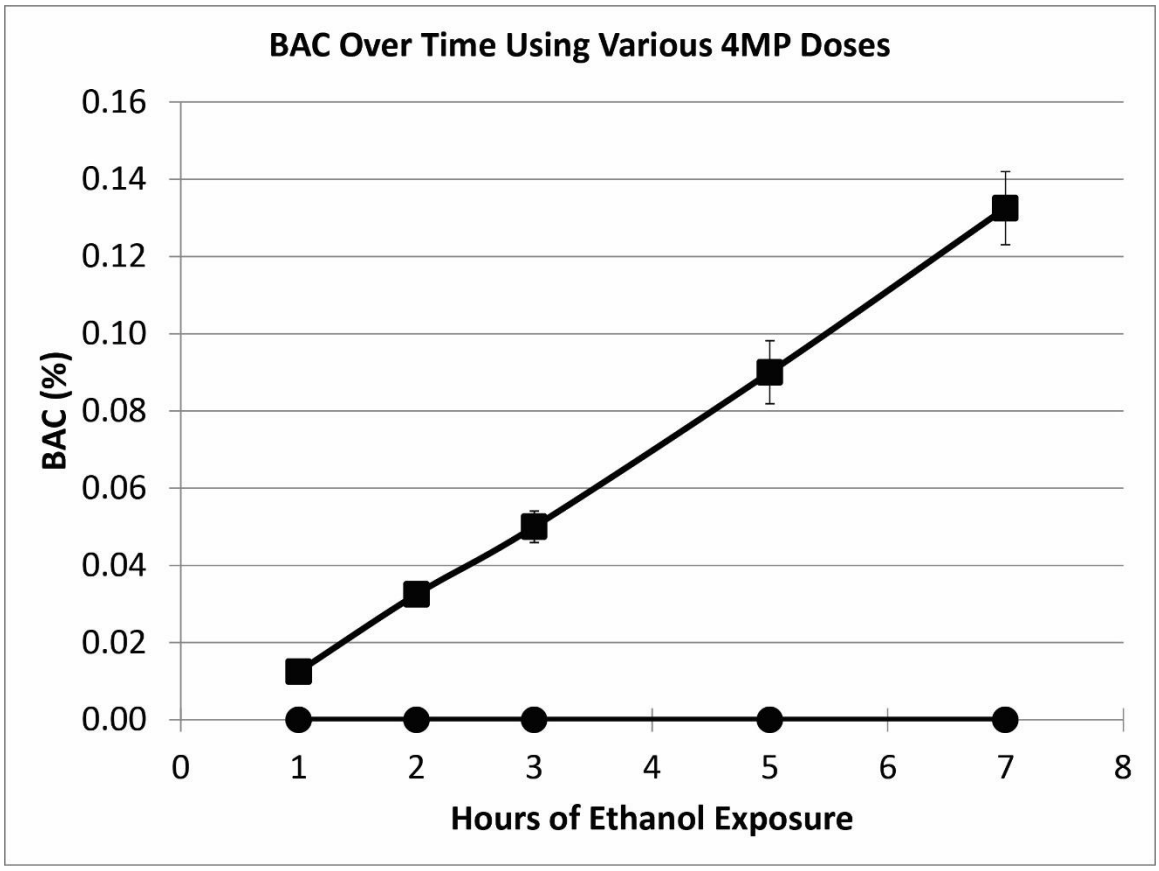

Figure 3.13: High-dose 4MP on BAC during vapor exposure.

A group of 5 rats weighing an average of 590 g received various high doses of 4MP (10 – 164 mg/kg), with one rat receiving only vehicle, and were then placed in low concentrations of ethanol vapor (15-20 mg/L). All rats had very similar BAC trajectories.

and 16 hours (Figure 3.14). The individual values at the 7-hour point from Figure 3.13 are shown as the "0-hour" point in Figure 3.14. By stabilizing ethanol metabolism rates, I was able to obtain reliable and predictable BAC's in my rodents.

With some clarity regarding the contribution of ethanol metabolism rate and a predictable escalation of BAC in vapor with doses as low as 10 mg/kg, my next goal was to determine the lowest dose that would allow predictable escalation while wearing off in time to allow ethanol to be cleared before the next exposure began. Using 5 of the "young" animals, now at 477 ± 33 g, I gave them various low doses of 4MP (5, 2.5, 1, 1, and 0.5 mg/kg) IP one hour before placing them into the same low-dose ethanol vapor (15-20 mg/L). I measured their BAC after 4, 7, and 14 hours of vapor exposure (Figure 3.15). After 14 hours of vapor exposure, I pulled them out of vapor and put them into fresh bedding and measured their BAC after 10 more hours. Notice that the 5 mg/kg and 2.5 mg/kg of pyrazole led to similar BAC after 7 hours as the high-dose animals in Figure 3.13, and both of those animals cleared their ethanol 10 hours later. Finally, I was able to reliably bring animals to within my target BAC of 0.15 – 0.20 %!

Although predictable BAC's were obtained with the use of 4MP, matching the animal weights and vapor concentrations used for the previous experiments would help answer questions about the contributions of ethanol metabolism rate to BAC while in vapor and test the theoretical model. Particular value would be gained from an experiment using 4MP in large versus small/adolescent rodents. Since MCP-1 or CCR2 antagonist

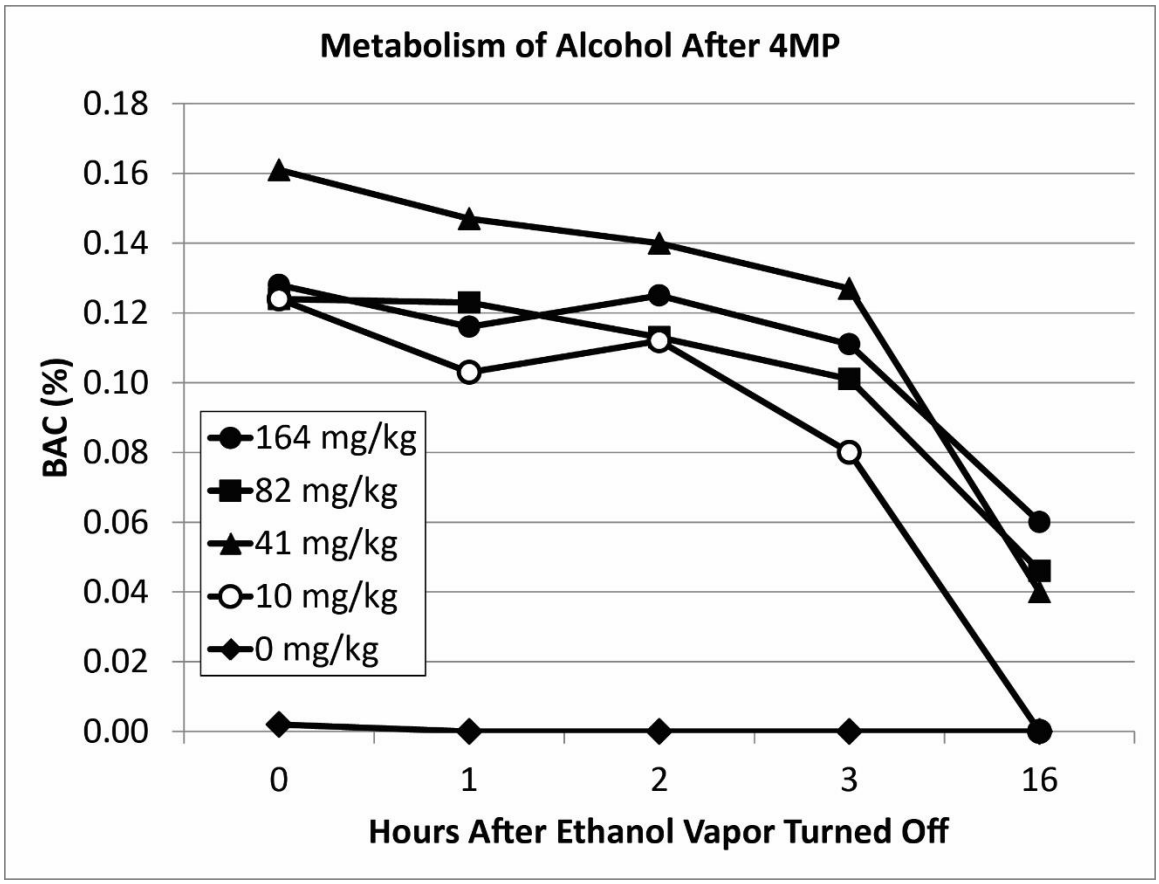

Figure 3.14: Metabolism of ethanol after various high doses of 4MP.

Individual BAC's after being pulled out of vapor and placed into fresh bedding. The lowest dose of 4MP (10 mg/kg) allowed the ethanol to be cleared by 16 hours.

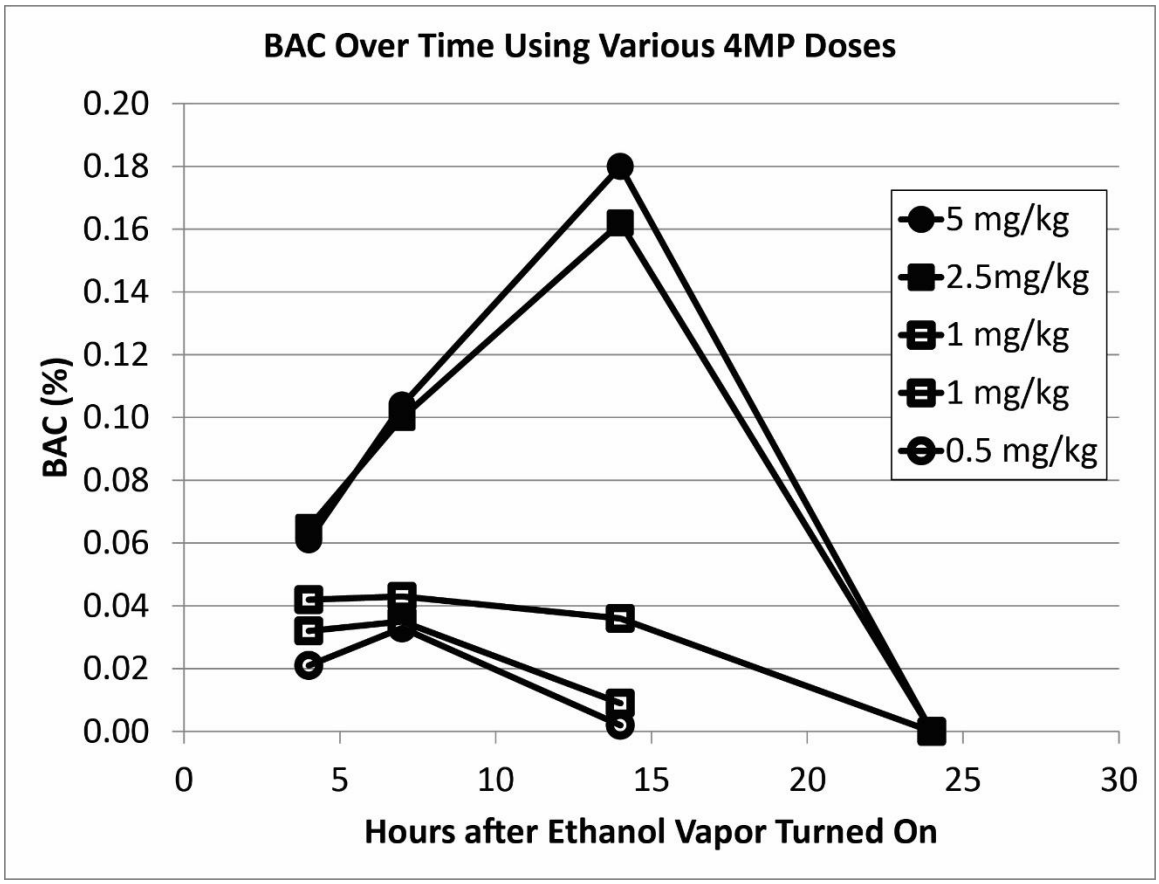

Figure 3.15: BAC after various low doses of 4MP.

Individual BAC's of "young" animals (now 477 g) exposed to 14 hours of ethanol vapor (15-20 mg/L) and then pulled out of vapor and put into fresh bedding for 10 more hours.

experiments would involve the use of osmotic minipumps for chronic administration, filling the minipumps with a cocktail of pyrazole and MCP-1 or CCR2 antagonist could be one way to avoid daily pyrazole injections but would likely lead to animals not clearing their BAC during the "off" period, so a continuous exposure model would have to be considered, such as the one used in O'Dell et al. (2004). Nonetheless, the use of Long-Evans rats in vapor exposure models seems to be more complicated than using Wistar or P rats (Gilpin et al., 2008; Gilpin et al., 2008b).

HOME CAGE DRINKING MODEL

Animal models of alcohol abuse that are characterized by an escalation in voluntary alcohol intake to intoxicating blood alcohol levels offer a valuable research tool for scientists. Our lab has put considerable effort into finding a suitable model of home-cage drinking without the use of sweetener. Although operant models of self-administration, which require rodents to press a lever to gain access to an ethanol solution, have more face validity as an animal model of human alcohol consumption, home cage models offer the advantage of being higher-throughput since they aren't limited by the number of operant chambers in the lab. I was interested in developing a reliable model of limited-access (30 minute) home cage drinking to maximize the throughput of experiments using ethanol vapor inhalation to escalate drinking. As previously mentioned, animals that become dependent on ethanol through vapor inhalation reliably escalate their ethanol intake during the 10-hour withdrawal periods. Several labs have used operant models to test escalations

in vapor dependence-induced drinking, but I have only found one other publication that used ethanol vapor to escalate home cage drinking (Sommer et al., 2008). My goal was to administer MCP-1 or the antagonist, ICV, to influence dependence-induced escalations in home cage drinking.

Several types of voluntary ethanol intake models exist, with variations occurring in ethanol access (e.g. continuous or intermittent), rodent strain, age of onset, and percentage of ethanol in the solution. Our lab was interested in replicating a common model of home cage, intermittent (MWF), 24-hour, 2-bottle choice ethanol access design in young adult male Long-Evans rats using a 20% ethanol solution. Typically, experiments performed using these criteria will result in rodents drinking 5–6 g/kg after 3 to 4 weeks, with 20% of rodents falling below a 3.5 g/kg cutoff (Simms et al., 2008; Carnicella et al., 2008; Carnicella et al., 2009; Carnicella et al., 2010; Ahmadiantehrani et al., 2011; Barak et al., 2011; Nielsen et al., 2012; Hwa et al., 2013; Meyer et al., 2013; Li et al., 2012). After establishing stable drinking levels, we would then use limited access sessions to teach the rodents to drink within a short time-frame in order to maximize blood alcohol levels and to allow experimental manipulation during specific time frames. These sessions would typically last 30 minutes or 1 hour. We also tried using a 15% ethanol solution. Ethanol concentration can be critical; if the concentration is too high, the taste is aversive. Many labs report 15% or 20% ethanol lead to the highest amounts of ethanol consumption. Although some rats can benefit from having the ethanol concentration escalated, we maintained the rodents in these experiments on a constant concentration of ethanol.

Comparing three replications using 20% ethanol (total n=20) with two replications using 15% ethanol (total n=12), during the first 12 sessions, the 20% groups escalated their intake faster than the 15% groups ($F_{11,327} = 1.85$, $P<0.05$). During week 4, the 20% groups averaged 3.01 ±0.24 g/kg while the 15% groups averaged 1.88 ±0.24 g/kg ($P<0.005$). Only a fraction of our animals (28%) reached the typical cutoff of 3.5 g/kg during the 4th week. After reaching stable drinking levels, a subset of animals were given limited-access to ethanol (60 minutes). Drinking levels peaked near 0.7 to 0.8 g/kg for all groups. Although our data did not replicate previously published data by other labs (in terms of max g/kg achieved), it was reliable enough to move forward.

I replicated the home cage experiment with a large cohort of young adult animals (starting at 200g), using only 20% ethanol. Initially, rodents were given 24-hour access on M/W/F, with 29 rodents having 24-hour access for 11 sessions before being switched over to 1-hour access, while 17 of the 29 had 24-hour access for 15 sessions before being switched over to 1-hour access (Figure 3.16). 1-hour access was measured the entire time, but bottles were immediately put back during 24-hour access sessions. The first day of 1-hour-only access corresponds to session 16 for all animals. Although not reaching the g/kg typically seen in the literature during 24-hour access, my values generally matched those of the lab's 20% experiments described above. Although these animals were not chronically exposed to ethanol vapor, a few were exposed for up to 6 sessions (previously shown in Figure 3.7). The recommended next step would be to determine if these animals would reliably escalate

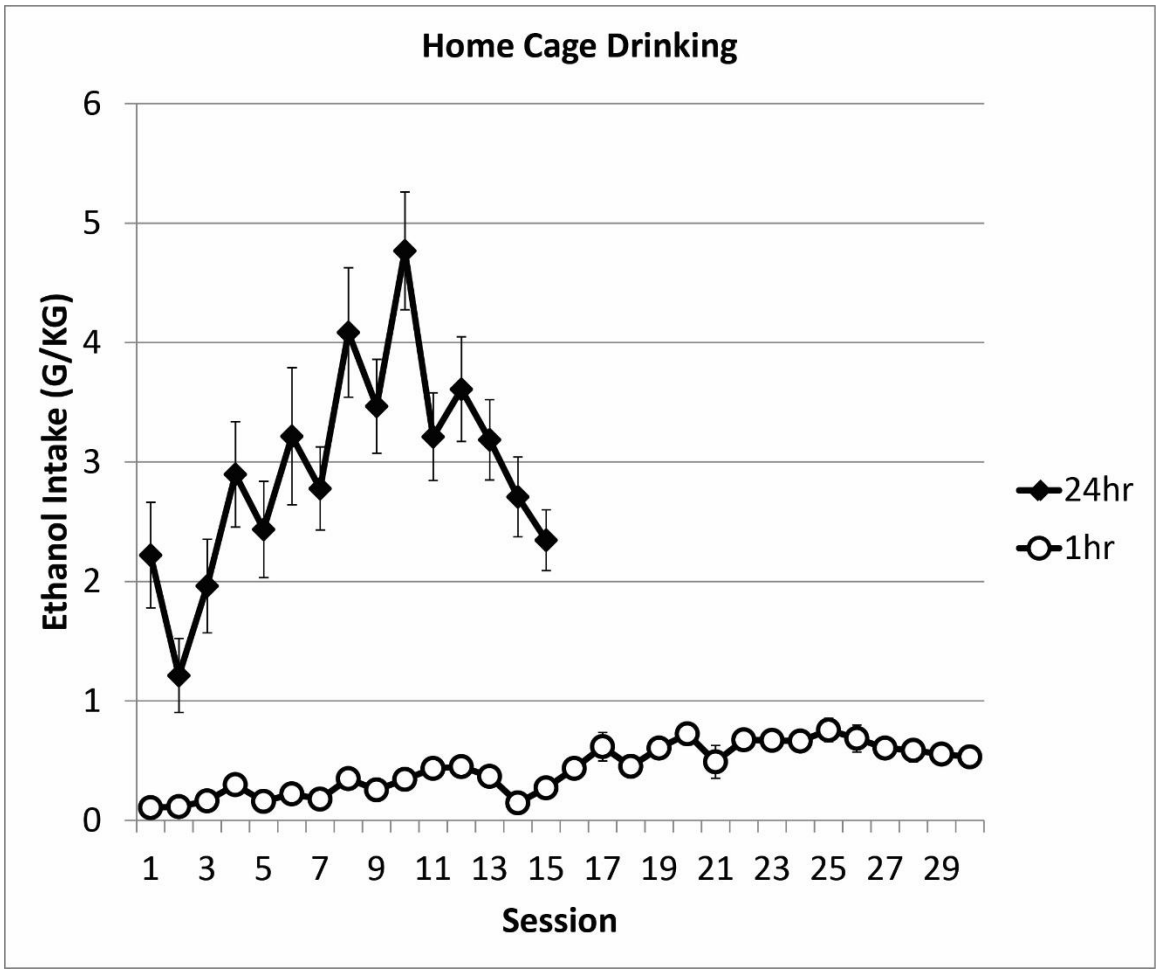

Figure 3.16: Home cage drinking experiment.

A cohort of 29 male Long-Evans rats weight approximately 200 g had access to ethanol in their home cage for 24 hours on M/W/F. Ethanol consumption was measured at 1 hour and at 24 hours. After 11 or 15 sessions, animals were switched to 1-hour access only. The first day of 1-hour access starts on session 16 for all animals in this graph for illustrative purposes.

their limited-access home cage drinking while becoming dependent on alcohol through chronic intermittent alcohol vapor exposure.

CONCLUSIONS

This chapter described my efforts to induce ethanol dependence in animals using repeated ethanol vapor exposures, as well as my efforts to develop a home cage ethanol drinking model. The goal of these experiments were to reliably increase home cage drinking in dependent animals and then manipulate drinking by using MCP-1 or the CCR2 antagonist.

I was not able to obtain BAC's within my target range using ethanol vapor for consecutive days unless 4MP was used to block alcohol metabolism. It seems that individual differences in ethanol metabolic rates contributes to variability in BAC in ethanol vapor (although, it is not the only variable). Ethanol metabolic rates seem to increase as the animals aged, as well as with repeated bouts of high BAC-inducing exposures. However, these increases were unpredictable. It is unclear if the increase in ethanol metabolic rate seen when the animals aged was simply due to the increase in the size of the liver. The increase was seen up to 325 g, but animals that were 500 g had slower ethanol metabolic rates. Further experimentation will be necessary to parse out the contributions of weight, liver size, and metabolic tolerance to BAC. However, reliable

BAC's are obtained when using 4MP, and experiments to induce dependence can move forward as long as 4MP is injected daily.

Chapter 5: Discussion and Conclusion

The goals of my dissertation project were to investigate the role of MCP-1 in ethanol self-administration, in animal models of both dependent and non-dependent ethanol self-administration. The initial plan was to investigate both the agonist and antagonist, but due to a variety of factors, this turned out to be over-ambitious. I have presented my progress toward these goals, which included experiments investigating the role of MCP-1 in a non-dependent model of ethanol self-administration, the development of a limited-access home cage drinking model, and obtaining reliable BAC's during ethanol vapor inhalation. This work spanned approximately 3 years, from the Fall of 2012 to the Fall of 2015. The first 3 years of my graduate study from the Fall of 2009 to the Fall of 2012, culminated in a first-author paper entitled "μ-opioid receptors in the stimulation of mesolimbic dopamine activity by ethanol and morphine in Long-Evans rats: a delayed effect of ethanol" which was published in the journal Psychopharmacology (Berl) in 2013.

In this chapter, I will present additional discussion that was not included in the published MCP-1 manuscript and concluding remarks.

DOPAMINE

One pervasive theme throughout this dissertation is the role of dopamine in drug abuse as well as the influence of neuroimmune signaling on dopamine neurotransmission.

Further discussion of this interaction is warranted, but a more formal introduction is necessary.

Mesolimbic dopamine neurons, which originate in the ventral tegmental area (VTA) and project to the ventral striatum, are thought to play a role in goal-directed behaviors, including operant self-administration and ethanol reinforcement (for review, see Gonzales et al., 2004). Activities that enhance the "survival of the species," such as food, water, and sex, which are referred to as "natural reinforcers", are powerful activators of mesolimbic dopamine neurons. Natural reinforcers and most drugs of abuse are known to increase dopamine release in the nucleus accumbens, primarily in the "shell" region of the nucleus accumbens. However, many drugs of abuse can increase dopamine neurotransmission to a far greater extent than the aforementioned natural reinforcers. Historically, mesolimbic dopamine was thought to act as a reward mechanism, serving as a hedonic indicator (Wise and Bozart, 1987). However, more recent data is suggesting that mesolimbic dopamine serves more as a predictor of reward than a rewarding mechanism in itself, determining the motivation in response to a particular context (Schultz et al., 1997; Schultz et al., 2007). For example, in animals that have previously learned to administer drugs of abuse, the dopamine increase is seen in response to the presentation of the drug rather than the intake of the drug itself (Schultz et al., 1997). Even though the precise function of dopamine neurons is still under debate (for a review, see Salamone and Correa, 2002), there is evidence that dopamine release is an essential component in the development of addictive behaviors, including drug abuse.

DOPAMINE AND ALCOHOL

Evidence gathered through pharmacological manipulation, genetic modification, and direct measurement support the importance of dopamine in many aspects of ethanol-seeking behavior. Data from our lab and many others have shown a transient increase in dopamine (~30%) during operant self-administration of ethanol (Doyon et al., 2003). Also, dopamine agonists can increase alcohol self-administration and the blockade of dopamine receptors can suppress ethanol reinforcement (Hodge et al., 1992; Rassnick et al., 1992). Furthermore, genetic knockout of dopamine receptors inhibits ethanol drinking (Phillips et al., 1998) and seeking behavior (Czachowski et al., 2001) while self-administration of ethanol directly into the nucleus accumbens has been reported in high-drinking rodent strains (Gatto et al., 1994; Rodd-Henricks et al., 2000; Engleman et al., 2009).

For drugs like cocaine and heroin, the cellular mechanisms that result in increased dopamine neurotransmission are fairly well understood (Johnson & North, 1992; Giros et al., 1996). However, the mechanism behind ethanol-stimulated dopamine release isn't as clear. Electrophysiological recordings provide evidence that ethanol can increase the firing rate of dopamine neurons in vivo and in vitro (Gessa et al., 1985; Brodie et al., 1999; Okamoto et al., 2006). However, the molecular mechanisms leading to that increase are still being sorted out. Nonetheless, it's clear that dopamine is important in self-administration, including the self-administration of ethanol.

MCP-1 AND DOPAMINE

Chemokines can influence neurotransmission through a variety of mechanisms, including through the modulation of neurotransmitter receptors or neurotransmitter release itself (Gosselin et al., 2005; Rostene et al., 2007; Guyon et al., 2009). As previously mentioned, MCP-1 increases dopamine release (Guyon et al., 2009) likely due to MCP-1 modulation of potassium channels (Guyon et al, 2009; Apartis et al, 2010; Wakida et al., 2014). A 50 ng bolus ICV injection also resulted in an increase in phosphorylated tyrosine hydroxylase levels in the VTA 24 hours later, while a CCR2 antagonist attenuated the conditioned place preference for methamphetamine (Wakida et al., 2014). How exactly MCP-1 influences dopamine release is still unclear, and may involve CCR2 activation of intracellular cascades that could influence potassium channel inactivation or internalization. Interestingly, the effect of MCP-1 on dopamine neurons is delayed several minutes, while wash-out effects are much faster (Guyon et al., 2009). Nonetheless, further experimentation is warranted to figure out these details.

CRF INCREASES MCP-1 SIGNALING IN THE VTA

As previously mentioned, stress hormones are thought to play a major role in the transition to alcohol dependence (for a review, see Heilig and Koob, 2007). The link between neuroimmune signaling and alcohol use disorders grew even stronger recently

with the discovery that CRF-induced increases in drinking in P rats are promoted by MCP-1 signaling in neurons of the VTA and CeA (June et al. 2015). Furthermore, June et al. (2015) also noted that P rats (a strain of inbred high alcohol preferring rats) have innately elevated levels of MCP-1 and TLR4 that colocalize in the neurons of those regions (compared to non-preferring "NP" rats). The interaction between stress and immune signaling is a fertile ground for the development of treatments for alcohol use disorders.

NEUROIMMUNE AMPLIFICATION

Alcohol, other drugs of abuse, stress, and immune signaling are all known to influence synaptic function, neuronal excitability, and influence reward system function. Furthermore, all of these also (further) increase neuroimmune gene expression or signaling. This has led to several researchers forming the hypothesis that neuroimmune loops drive addiction (Crews et al., 2011; Mayfield et al., 2013; Cui et al., 2014). A simplified schematic highlighting the role of MCP-1 and TNFα in the propagation of neuroimmune gene induction and neurotoxicity is shown in Figure 5.1 (adapted from Zou and Crews, 2010). Neuroimmune influences on all three stages of addiction, including binge/intoxication, withdrawal/negative affect, and preoccupation/anticipation can all be amplified with repeat exposures and lead to persistent effects. Influences on reward circuitry, stress systems, executive control, and anxiety circuitry are all common denominators. Thus, neuroimmune activation/amplification may be a critical component of the brain dysfunction that drives addictive behaviors.

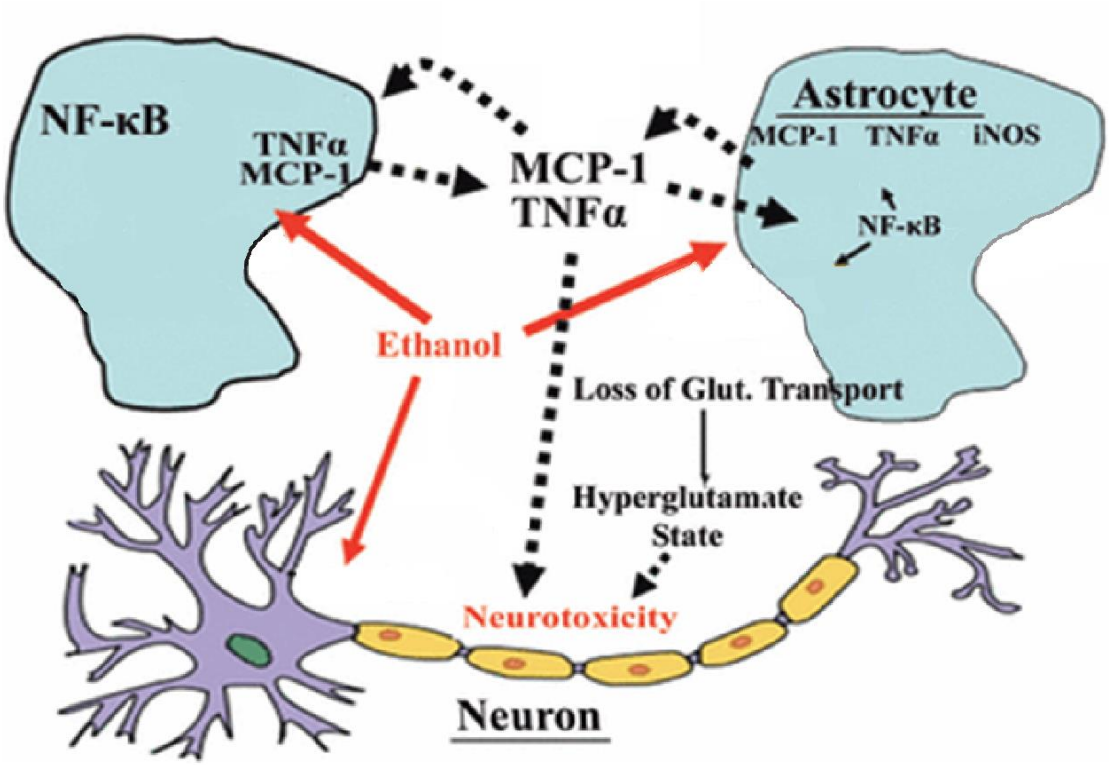

Figure 5.1: Amplification of MCP-1 signaling

Schematic highlighting the role of MCP-1 and TNFα in the propagation of neuroimmune gene induction and neurotoxicity. Microglia, astrocytes, and neurons can all secrete MCP-1 and have CCR2 receptors. Microglia can amplify their own recruitment to a site of brain injury. This phenomenon may also drive progression through the stages of addiction. Adapted from Zou and Crews (2010) with permission.

A variety of immune receptor signaling cascades lead to the activation of the activator protein 1 (AP-1) and NF-kB transcription factors, which induce cell-specific gene transcription. In microglia, activation of the CCR2 receptor is known to induce NF-kB (See Figure 1.2). NF-kB is constitutively transcriptionally active in the brain and is central to the promotion of a neuroinflammatory response, including the transcription of MCP-1 and various other cytokines. Activated microglia or astrocytes, neurons, and infiltrating leukocytes will secrete additional cytokines, including MCP-1 (for a review, see Semple et al., 2010), resulting in an amplification of signaling (Cushing and Fogelman, 1991; Tieu et al., 2009; Gunn et al., 1997). Furthermore, activated microglia, astrocytes, and neurons all express CCR2 receptors. Evidence suggests that monocytes amplify their own recruitment to an inflammatory site, and subsequent activation, through autocrine induction of MCP-1 (Cushing and Fogelman, 1991).

This is particularly noteworthy because ethanol has be shown to cause the release of MCP-1 (Zou and Crews, 2010; Qin et al., 2008; He and Crews, 2008). Additionally, ethanol itself, under specific conditions, is known to induce microglial activation, although which stage of activation is under debate (Marshall et al., 2013; Crews et al., 2006). Given that our data has shown an increase in ethanol self-administration due to MCP-1 infusion, an effect that sensitizes over several weeks of drinking and results in persistent effects, it's possible that a feed-forward mechanism is driving the increase in drinking. A feed-forward mechanism could be tested by measuring MCP-1 in the brains of these animals at various

points in time throughout the experiment. The hypothesis that ethanol-induced increases in MCP-1 may drive progression through the stages of addiction is ripe for investigation.

LIMITATIONS

Given that MCP1/CCR2 knockout mice drink significantly less alcohol than controls (Figure 1.3), experiments using a CCR2 antagonist are justified. The ability of CCR2 antagonists to modulate ethanol drinking behavior has never been investigated. The osmotic minipump is particularly well-suited for the delivery of the antagonist. Several experiments come to mind. One would be to test the ability of the antagonist in a binge or non-dependent self-adnimistration model. Another would be to prevent the escalation of drinking due to vapor exposure. The goal would be to completely block MCP-1 signaling continuously throughout the development of dependence in order to make sure that transient MCP-1 signaling doesn't occur. A third experiment would be to investigate the therapeutic potential of the antagonist by administering it to already-dependent rodents.

The specific brain regions involved in the MCP-1 experiments was not determined because ICV infusions are known to distribute throughout the brain. Determining which brain regions involved in the neuroimmune regulation of drinking behavior will increase our understanding of the phenomenon and can lead to more selective compounds with fewer potential side effects. The mesolimbic dopamine system has been identified as a candidate due to its importance in the regulation of drinking behavior and the presence of

CCR2 receptors on those neurons. Microinjections of either MCP-1 or the antagonist into the ventral tegmental area would be warranted.

CONCLUSION

The results of my experiments provide critical new knowledge about a new area of research. By understanding how neuroimmune signaling can facilitate the acquisition or escalation of drinking behavior, we can develop insights into the transition from healthy to unhealthy drinking behavior and we can develop new molecular targets for treatment. Also, once we identify the key molecules involved, we could develop means of identifying those at high risk for the disease and take precautionary measures to help them avoid developing the disease, including monitoring or prophylactic treatment. Furthermore, advances in therapeutics for alcohol abuse can potentially translate into therapeutics for substance abuse in general. Our results will help motivate investigations into whether neuroimmune signaling can be used for the treatments for other behavioral pathologies. Finally, our data may have a broader scientific significance by supporting theories that complex behaviors are regulated by neuroimmune signaling.

References

Andersson U, Tracey KJ (2011). HMGB1 is a therapeutic target for sterile inflammation and infection. Annu Rev immunol 29:139-162.

Agrawal RG, Hewetson A, George CM, Syapin PJ, Bergeson SE (2011). Minocycline reduces ethanol drinking. Brain Behav Immun 25 Suppl 1:S165-9.

Ahmadiantehrani, S, Barak S, Ron D (2013). GDNF is a novel ethanol-responsive gene in the VTA: implications for the development and persistence of excessive drinking. Addiction Biology, 19, 623-633.

Alfonso-Loeches S, Pascual-Lucas M, Blanco AM, Sanchez-Vera I, Guerri C (2010) Pivotal role of TLR4 receptors in alcohol-induced neuroinflammation and brain damage. J Neurosci 16; 30(24):8285-95.

Apartis E, Mélik-Parsadaniantz S, Guyon A, Kitabgi P, Rostène W (2010) Chemokines as new actors in the dopaminergic system. Biol Aujourdhui 204(4):295-300.

Baamonde A, Hidalgo A, Menéndez L (2011). Involvement of glutamate NMDA and AMPA receptors, glial cells and IL-1 β in the spinal hyperalgesia evoked by the chemokine CCL2 in mice. Neurosci Lett 502(3):178-81.

Banisadr G, Quéraud-Lesaux F, Boutterin MC, Pélaprat D, Zalc B, Rostène W, Haour F, Parsadaniantz SM (2002) Distribution, cellular localization and functional role of CCR2 chemokine receptors in adult rat brain. J Neurochem 81(2):257-69

Banisadr G, Gosselin RD, Mechighel P, Rostène W, Kitabgi P, Mélik Parsadaniantz S (2005). Constitutive neuronal expression of CCR2 chemokine receptor and its colocalization with neurotransmitters in normal rat brain: functional effect of MCP-1/CCL2 on calcium mobilization in primary cultured neurons. J Comp Neurol 492(2):178-92.

Banisadr G, Gosselin RD, Mechighel P, Kitabgi P, Rostène W, Parsadaniantz SM (2005b). Highly regionalized neuronal expression of monocyte chemoattractant protein-1 (MCP-1/CCL2) in rat brain: evidence for its colocalization with neurotransmitters and neuropeptides. J Comp Neurol 489(3):275-92.

Barak S, Ahmadiantehrani S, Kharazia V, Ron D (2011). Positive autoregulation of GDNF levels in the ventral tegmental area mediates long-lasting inhibition of excessive alcohol consumption. Transl Psychiatry 1: pii: e60.

Barrientos RM, Higgins EA, Sprunger DB, Watkins LR, Rudy JW, Maier SF (2002). Memory for context is impaired by a post context exposure injection of interleukin-1 beta into dorsal hippocampus. Behav Brain Res 134(1-2):291-8.

Bell MD, Taub DD, Perry VH (1996) Overriding the brain's intrinsic resistance to leukocyte recruitment with intraparenchymal injections of recombinant chemokines. Neuroscience 74(1):283-92.

Besedovsky H, Sorkin E, Felix D, Haas H (1977). Hypothalamic changes during the immune response. Eur. J. Immunol. 7: 323-325.

Blalock JE (1989). A molecular basis for bidirectional communication between the immune and neuroendocrine systems. Physiol Rev. 69: 1-32.

Blanco AM, Guerri C (2007). Ethanol intake enhances inflammatory mediators in brain: role of glial cells and TLR4/IL-1RI receptors. Front Biosci 12:2616-30.

Bland ST, Hutchinson MR, Maier SF, Watkins LR, Johnson KW (2009). The glial activation inhibitor AV411 reduces morphine-induced nucleus accumbens dopamine release. Brain Behav Immun 23(4):492-7.

Blednov YA, Bergeson SE, Walker D, Ferreira VM, Kuziel WA, Harris RA (2005) Perturbation of chemokine networks by gene deletion alters the reinforcing actions of ethanol. Behav Brain Res 165(1):110-25.

Blednov YA, Benavidez JM, Geil C, Perra S, Morikawa H, Harris RA (2011) Activation of inflammatory signaling by lipopolysaccharide produces a prolonged increase of voluntary alcohol intake in mice. Brain Behav Immun 25 (1):92-105.

Blednov YA, Ponomarev I, Geil C, Bergeson S, Koob GF, Harris RA (2011b). Neuroimmune regulation of alcohol consumption: behavioral validation of genes obtained from genomic studies. Addict Biol 17(1):108-20.

Blednov YA, Ponomarev I, Geil C, Bergeson S, Koob GF, Harris RA (2012). Neuroimmune regulation of alcohol consumption: behavioral validation of genes obtained from genomic studies. Addict Biol 17(1):108-20.

Block ML, Zecca L, Hong JS (2007). Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. Nat Rev Neurosci 8(1):57-69.

Blomstrand R, Ostling H (1977). Interactions between ethanol and 4-methyl pyrazole. In: R.G. Thurman, J.R. Williamsson, H.R. Drott, B. Chance (Eds.) Alcohol and Aldehyde Metabolizing Systems. Vol III. Academic Press, New York; 1977:427–436.

Bloom F, Lad P, Pittman Q, Rogers J (1982). Blood alcohol levels in rats: non-uniform yields from intraperitoneal doses based on body weight. Br J Pharmacol 75:251–254.

Borovikova LV, Ivanova S, Zhang M, Yang H, Botchkina GI, Watkins LR, Wang H, Abumrad N, Eaton JW, Tracey KJ (2000). Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. Nature 405(6785):458-62.

Bouchery EE, Harwood HJ, Sacks JJ, Simon CJ, Brewer RD (2011). Economic costs of excessive alcohol consumption in the U.S., 2006. Am J Prev Med 41(5):516-24.

Breder CD, Hazuka C, Ghayur T, Klug C, Huginin M, Yasuda K, Teng M, Saper CB (1994). Regional induction of tumor necrosis factor alpha expression in the mouse

brain after systemic lipopolysaccharide administration. Proc Natl Acad Sci 91(24):11393-7.

Breder CD, Dinarello CA, Saper CB (1998). Interleukin-1 immunoreactive innervation of the human hypothalamus. Science 240, 321-324.

Breese GR, Knapp DJ, Overstreet DH (2004) Stress sensitization of ethanol withdrawal-induced reduction in social interaction: inhibition by CRF-1 and benzodiazepine receptor antagonists and a 5-HT1A-receptor agonist. Neuropsychopharmacology 29(3):470-82.

Breese GR, Knapp DJ, Overstreet DH, Navarro M, Wills TA, Angel RA (2008) Repeated lipopolysaccharide(LPS) or cytokine treatments sensitize ethanol withdrawal-induced anxiety-like behavior. Neuropsychopharmacology 33(4):867-76.

Breese GR, Sinha R, Heilig M (2011). Chronic alcohol neuroadaptation and stress contribute to susceptibility for alcohol craving and relapse. Pharmacol Ther 129(2): 149-171.

Brodie MS, Pesold C, Appel SB (1999). Ethanol directly excites dopaminergic ventral tegmental area reward neurons. Alcohol Clin Exp Res 23:1848-1852.

Callewaere C, Banisadr G, Desarménien MG, Mechighel P, Kitabgi P, Rostène WH, Mélik Parsadaniantz S (2006).The chemokine SDF-1/CXCL12 modulates the firing pattern of vasopressin neurons and counteracts induced vasopressin release through CXCR4. Proc Natl Acad Sci USA 103(21):8221-6.

Capotondo A, Milazzo R, Politi LS, Quattrini A, Palini A, Plati T, Merella S, Nonis A, di Serio C, Montini E, Naldini L, Biffi A (2012). Brain conditioning is instrumental for successful microglia reconstitution following hematopoietic stem cell transplantation. Proc Natl Acad Sci 109(37):15018-23.

Carnevale D, De Simone R, Minghetti L (2007). Microglia-neuron interaction in inflammatory and degenerative diseases: role of cholinergic and noradrenergic systems. CNS Neurol Disord Drug Targets 6(6):388-97.

Carnicella S, Kharazia V, Jeanblanc J, Janak PH, Ron D (2008). GDNF is a fast-acting potent inhibitor of alcohol consumption and relapse. Proc Natl Acad Sci 105(23):8114-9.

Carnicella S, Amamoto R, Ron D (2009). Excessive alcohol consumption is blocked by glial cell line-derived neurotrophic factor. Alcohol, 43, 35-43.

Carnicella S, He DY, Yowell QV, Glick SD, Ron D (2010). Noribogaine, but not 18-MC, exhibits similar actions as ibogaine on GDNF expression and ethanol self-administration. Addiction Biology 15, 424-433.

Carrillo J, Gonzales RA (2011) A single exposure to voluntary ethanol self-administration produces adaptations in ethanol consumption and accumbal dopamine signaling. Alcohol 45:559–566.

Cederbaum A (2012). Alcohol metabolism. Clin Liver Dis 16(4):667-85.

Collins AC, Yeager TN, Lebsack ME, Panter SS (1975). Variations in alcohol metabolism: influence of sex and age. Pharmacol Biochem Behav 3(6):973-8.

Colton CA (2009). Heterogeneity of microglial activation in the innate immune response in the brain. J Neuroimmune Pharmacol 4(4): 399-418.

Conductier G, Blondeau N, Guyon A, Nahon JL, Rovère C (2010). The role of monocyte chemoattractant protein MCP1/CCL2 in neuroinflammatory diseases. J Neuroimmunol 224(1-2):93-100.

Crews F, K Nixon, Kim D, Joseph J, Shukitt-Hale B, Qin L, Zou J (2006). BHT blocks NF-kappaB activation and ethanol-induced brain damage. Alcohol Clin Exp Res 30(11): 1938-1949.

Crews FT, Nixon K (2009). Mechanisms of neurodegeneration and regeneration in alcoholism. Alcohol 44, 115–127.

Crews FT, Zou J, Qin L (2011). Induction of innate immune genes in brain create the neurobiology of addiction. Brain Behav Immun 25 Suppl 1: S4-S12.

Crews FT, Qin L, Sheedy D, Vetreno RP, Zou J (2012). High mobility group box 1/Toll-like receptor danger signaling increases brain neuroimmune activation in alcohol dependence. Biol Psychiatry 73(7):602-12.

Cui C, Shurtleff D, Harris RA (2014). Chapter One - Neuroimmune Mechanisms of Alcohol and Drug Addiction. In: Cui C, Shurtleff D, Harris RA (Eds.) Neuroimmune Signaling in Drug Actions and Addictions Vol 118. Academic Press, London: 2014: 1-12.

Cushing SD, Fogelman AM (1991) Monocytes may amplify their recruitment into inflammatory lesions by inducing monocyte chemotactic protein. Arterioscler Thromb 12(1):78-82.

Czachowski CL, Chappell AM, Samson HH (2001) Effects of raclopride in the nucleus accumbens on ethanol seeking and consumption. Alcohol Clin Exp Res 25:1431-1440.

Dansereau MA, Gosselin RD, Pohl M, Pommier B, Mechighel P, Mauborgne A, Rostene W, Kitabgi P, Beaudet N, Sarret P, Melik-Parsadaniantz S (2008). Spinal CCL2 pronociceptive action is no longer effective in CCR2 receptor antagonist-treated rats. J Neurochem 106(2):757-69.

Dantzer R, O'Connor JC, Freund GG, Johnson RW, Kelley KW (2008). From inflammation to sickness and depression: when the immune system subjugates the brain. Nat Rev Neurosci 9(1):46-56.

Deis, D (1979). Lester Biochemical pharmacology of pyrazoles. In: Biochemistry and Pharmacology of Ethanol, Vol. 2 (E. Majchrowicz and E.P. Noble, eds.), Plenum Press, New York (1979), pp. 303–323.

Domercq M, Vázquez-Villoldo N, Matute C (2011). Neurotransmitter signaling in the pathophysiology of microglia. Front Cell Neurosci 7:49.

Doyon WM, York JL, Diaz LM, Samson HH, Czachowski CL, Gonzales RA (2003). Dopamine activity in the nucleus accumbens during consummatory phases of oral ethanol self-administration. Alcohol Clin Exp Res 27(10):1573-82.

Dzenko KA, Andjelkovic AV, Kuziel WA, Pachter JS (2001) The chemokine receptor CCR2 mediates the binding and internalization of monocyte chemoattractant protein-1 along brain microvessels. J Neurosci 1; 21(23):9214-23.

Engleman EA, Ding ZM, Oster SM, Toalston JE, Bell RL, Murphy JM, McBride WJ, Rodd ZA (2009). Ethanol is self-administered into the nucleus accumbens shell, but not the core: evidence of genetic sensitivity. Alcohol Clin Exp Res 33:2162-2171.

Ehrlich D, Pirchl M, Humpel C (2012) Effects of long-term moderate ethanol and cholesterol on cognition, cholinergic neurons, inflammation, and vascular impairment in rats. Neuroscience 15; 205:154-66.

Ferko AP, Babyock E (1976) Induction of physical dependence in rats by ethanol inhalation without the use of pyrazole. Toxicol Appl Pharmacol 40(2):269-76.

Ferrari CC1, Tarelli R (2011). Parkinson's disease and systemic inflammation. Parkinsons Dis 2011:436813.

Flügel A, Hager G, Horvat A, Spitzer C, Singer GM, Graeber MB, Kreutzberg GW, Schwaiger FW (2001). Neuronal MCP-1 expression in response to remote nerve injury. J Cereb Blood Flow Metab 21(1):69-76.

Funk CK, O'Dell LE, Crawford EF, Koob GF (2006). Corticotropin-releasing factor within the central nucleus of the amygdala mediates enhanced ethanol self-administration in withdrawn, ethanol-dependent rats. J Neurosci 26(44):11324-32.

Funk CK, Zorrilla EP, Lee MJ, Rice KC, Koob GF (2007). Corticotropin-releasing factor 1 antagonists selectively reduce ethanol self-administration in ethanol-dependent rats. Biol Psychiatry 61(1):78-86.

Gass JT, Glen WB Jr, McGonigal JT, Trantham-Davidson H, Lopez MF, Randall PK, Yaxley R, Floresco SB, Chandler LJ (2014). Adolescent alcohol exposure reduces behavioral flexibility, promotes disinhibition, and increases resistance to extinction of ethanol self-administration in adulthood. Neuropsychopharmacology 39(11):2570-83.

Gatto GJ, McBride WJ, Murphy JM, Lumeng L, Li TK (1994). Ethanol self-infusion into the ventral tegmental area by alcohol- preferring rats. Alcohol 11:557-564.

Gehrmann J, Matsumoto Y, Kreutzberg GW (1995). Microglia: intrinsic immuneffector cell of the brain. Brain Res Brain Res Rev 20(3):269-87.

Gessa GL, Muntoni F, Collu M, Vargiu L, Mereu G (1985). Low doses of ethanol activate dopaminergic neurons in the ventral tegmental area. Brain Research 348:201-203.

Gilpin NW, Richardson HN, Lumeng L, Koob GF (2008). Dependence-induced alcohol drinking by alcohol-preferring (P) rats and outbred Wistar rats. Alcohol Clin Exp Res 32(9):1688-96.

Gilpin NW, Richardson HN, Cole M, Koob GF (2008b). Vapor inhalation of alcohol in rats. Curr Protoc Neurosci July: Chapter 9: Unit 9.29.

Gilpin NW, Smith AD, Cole M, Weiss F, Koob GF, Richardson HN (2009). Operant behavior and alcohol levels in blood and brain of alcohol-dependent rats. Alcohol Clin Exp Res 33(12):2113-23.

Ginhoux F, Lim S, Hoeffel G, Low D, Huber T (2013). Origin and differentiation of microglia. Front Cell Neurosci 7:45.

Giros B, Jaber M, Jones SR, Wightman RM, Caron MG (1996). Hyperlocomotion and indifference to cocaine and amphetamine in mice lacking the dopamine transporter. Nature 379, 606– 612.

Goldberg L, Rydberg U (1969). Inhibition of ethanol metabolism in vivo by administration of pyrazole. Biochem Pharmacol 18(7):1749-62.

Gonzales RA, Job MO, Doyon WM (2004). The role of mesolimbic dopamine in the development and maintenance of ethanol reinforcement. Pharmacol Ther 103:121–146.

Gosselin RD, Varela C, Banisadr G, Mechighel P, Rostene W, Kitabgi P, Melik-Parsadaniantz S (2005). Constitutive expression of CCR2 chemokine receptor and inhibition by MCP-1/CCL2 of GABA-induced currents in spinal cord neurones. J Neurochem 95(4):1023-34.

Gourmala NG, Buttini M, Limonta S, Sauter A, Boddeke HW (1997). Differential and time-dependent expression of monocyte chemoattractant protein-1 mRNA by astrocytes and macrophages in rat brain: effects of ischemia and peripheral lipopolysaccharide administration. J Neuroimmunol 74(1-2):35-44.

Graeber MB (2010). Changing face of microglia. Science 330, 783–788.

Gruber AJ, Calhoon GG, Shusterman I, Schoenbaum G, Roesch MR, O'Donnell P (2010). More is less: a disinhibited prefrontal cortex impairs cognitive flexibility. J Neurosci 30(50):17102-10.

Gunn MD, Nelken NA, Liao X, Williams LT (1997) Monocyte chemoattractant protein-1 is sufficient for the chemotaxis of monocytes and lymphocytes in transgenic mice

but requires an additional stimulus for inflammatory activation. J Immunol 1; 158(1):376-83.

Guyon A, Skrzydelski D, De Giry I, Rovère C, Conductier G, Trocello JM, Daugé V, Kitabgi P, Rostène W, Nahon JL, Mélik Parsadaniantz S (2009) Long term exposure to the chemokine CCL2 activates the nigrostriatal dopamine system: a novel mechanism for the control of dopamine release. Neuroscience 15; 162(4):1072-80.

Hasin DS, Stinson FS, Ogburn E, Grant BF (2007). Prevalence, correlates, disability, and comorbidity of DSMIV alcohol abuse and dependence in the United States: results from the National Epidemiologic Survey on Alcohol and Related Conditions. Arch Gen Psychiatry 64(7):830-42.

He J, Crews FT (2008) Increased MCP-1 and microglia in various regions of the human alcoholic brain. ExpNeurol 210(2):349-58.

Heilig M, Koob GF (2007). A key role for corticotropin-releasing factor in alcohol dependence. Trends Neurosci 30: 399–406.

Hinojosa AE, Garcia-Bueno B, Leza JC, Madrigal JL (2011) CCL2/MCP-1 modulation of microglial activation and proliferation. J Neuroinflammation 5; 8:77.

Hodge CW, Samson HH, Haraguchi M (1992). Microinjections of dopamine agonists in the nucleus accumbens increase ethanol reinforced responding. Pharmacol Biochem Behav 43, 249– 254.

Howard EC, Schier CJ, Wetzel JS, Gonzales RA (2009) The dopamine response in the nucleus accumbens core-shell border differs from that in the core and shell during operant ethanol self-administration. Alcohol Clin Exp Res 33(8):1355-65.

Hueston CM, Deak T (2014). The inflamed axis: The interaction between stress, hormones, and the expression of inflammatory-related genes within key structures comprising the hypothalamic–pituitary–adrenal axis. Physiol Behav 124: 77–91.

Holm TH, Draeby D, Owens T (2012). Microglia are required for astroglial Toll-like receptor 4 response and for optimal TLR2 and TLR3 response. Glia 60(4):630-8.

Hughes V (2012). Microglia: the constant gardeners. Nature 485, 570–572.

Hutchinson MR, Northcutt AL, Chao LW, Kearney JJ, Zhang Y, Berkelhammer DL, Loram LC, Rozeske RR, Bland ST, Maier SF, Gleeson TT, Watkins LR (2008). Minocycline suppresses morphine-induced respiratory depression, suppresses morphine-induced reward, and enhances systemic morphine-induced analgesia. Brain Behav Immun 22(8):1248-56.

Hwa LS, Debold JF, Miczek KA (2013). Alcohol in excess: CRF_1 receptors in the rat and mouse VTA and DRN. Psychopharmacology (Berl) 225(2):313-27.

Ishizuka K, Kimura T, Igata-yi R, Katsuragi S, Takamatsu J, Miyakawa T (1997). Identification of monocyte chemoattractant protein-1 in senile plaques and reactive microglia of Alzheimer's disease. Psychiatry Clin Neurosci 51(3):135-8.

Jourdain P, Bergersen LH, Bhaukaurally K, Bezzi P, Santello M, Domercq M, Matute C, Tonello F, Gundersen V, Volterra A (2007). Glutamate exocytosis from astrocytes controls synaptic strength. Nat Neurosci 10(3):331-9.

Johnson SW, North RA (1992). Opioids excite dopamine neurons by hyperpolarization of local interneurons. J Neurosci 12, 483–488.

June HL, Liu J, Warnock KT, Bell KA, Balan I, Bollino D, Puche A, Aurelian L (2015) CRF-Amplified Neuronal TLR4/MCP-1 Signaling Regulates Alcohol Self-Administration. Neuropsychopharmacology 40(6):1549-59.

Kettenmann H, Hanisch UK, Noda M, Verkhratsky A (2011). Physiology of microglia. Physiol. Rev. 91, 461–553.

Kimelberg HK, Jalonen T, Walz W (1993). Regulation of the brain microenvironment: transmitters and ions. In Murphy, S. Astrocytes: pharmacology and function. San Diego, CA: Academic Press. pp. 193–222.

Kimpel MW, Strother WN, McClintick JN, Carr LG, Liang T, Edenberg HJ, McBride WJ (2007). Functional gene expression differences between inbred alcohol-preferring and -non-preferring rats in five brain regions. Alcohol 41(2):95-132.

Kingwell,K (2012). Neurodegenerative disease: microglia in early disease stages. Nat Rev Neurol 8, 475.

Koob GF (2004). A role for GABA mechanisms in the motivational effects of alcohol. Biochem Pharmacol 15; 68(8):1515-25.

Lawson LJ1, Perry VH, Dri P, Gordon S (1990). Heterogeneity in the distribution and morphology of microglia in the normal adult mouse brain. Neuroscience 39(1):151-70.

Lawson LJ, Perry VH, Gordon S (1992). Turnover of resident microglia in the normal adult mouse brain. Neuroscience 48, 405–415.

Lehnardt S (2010). Innate immunity and neuroinflammation in the CNS: the role of microglia in Toll-like receptor-mediated neuronal injury. Glia 58(3): 253-263.

Li J, Nie H, Bian W, Dave V, Janak PH, Ye JH (2012). Microinjection of glycine into the ventral tegmental area selectively decreases ethanol consumption. J Pharmacol Exp Ther 341(1):196-204.

Liu J, Lewohl JM, Harris RA, Iyer VR, Dodd PR, Randall PK, Mayfield RD (2006). Patterns of gene expressionin the frontal cortex discriminate alcoholic from nonalcoholic individuals. Neuropsychopharmacology 31(7):1574-82.

Lotze MT, Tracey KJ (2005). High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. Nat Rev Immunol 5(4):331-42.

Madrigal JL, Moro MA, Lizasoain I, Lorenzo P, Leza JC (2002) Stress-induced increase in extracellular sucrose space in rats is mediated by nitric oxide. Brain Res 938(1-2): 87-91.

Maroso M, Balosso S, Ravizza T, Liu J, Aronica E, Iyer AM, Rossetti C, Molteni M, Casalgrandi M, Manfredi AA, Bianchi ME, Vezzani A (2010). Toll-like receptor 4 and high-mobility group box-1 are involved in ictogenesis and can be targeted to reduce seizures. Nat Med 16(4):413-9.

Marshall SA, McClain JA, Kelso ML, Hopkins DM, Pauly JR, Nixon K (2013). Microglial activation is not equivalent to neuroinflammation in alcohol-induced neurodegeneration: the importance of microglia phenotype. Neurobiol Dis 54:239-51.

Mayfield J, Ferguson L, Harris RA (2013). Neuroimmune signaling: a key component of alcohol abuse. Curr Opin Neurobiol (4):513-20.

Melgarejo E, Medina MA, Sánchez-Jiménez F, Urdiales JL (2009). Monocyte chemoattractant protein-1: a key mediator in inflammatory processes. Int J Biochem Cell Biol 41(5):998-1001.

Meyer EM, Long V, Fanselow MS, Spigelman I (2013). Stress increases voluntary alcohol intake, but does not alter established drinking habits in a rat model of posttraumatic stress disorder. Alcohol Clin Exp Res 37(4):566-74.

Monk PN, Shaw PJ (2006). ALS: life and death in a bad neighborhood. Nat Med 12(8):885-7.

Mrak RE, Griffin WS (2005). Glia and their cytokines in progression of neurodegeneration. Neurobiol Aging 26(3):349-54.

Mulligan MK, Ponomarev I, Hitzemann RJ, Belknap JK, Tabakoff B, Harris RA, Crabbe JC, Blednov YA, Grahame NJ, Phillips TJ, Finn DA, Hoffman PL, Iyer VR, Koob GF, Bergeson SE (2006). Toward understanding the genetics of alcohol drinking through transcriptome meta-analysis. Proc Natl Acad Sci 103(16):6368-73.

Munhoz CD, Lepsch LB, Kawamoto EM, Malta MB, Lima Lde S, Avellar MC, Sapolsky RM, Scavone C (2006). Chronic unpredictable stress exacerbates lipopolysaccharide-induced activation of nuclear factor-kappaB in the frontal cortex and hippocampus via glucocorticoid secretion. J Neurosci 26(14):3813-20.

Narita M, Miyatake M, Narita M, Shibasaki M, Shindo K, Nakamura A, Kuzumaki N, Nagumo Y, Suzuki T (2006). Direct evidence of astrocytic modulation in the development of rewarding effects induced by drugs of abuse Neuropsychopharmacology 31(11):2476-88.

Nielsen CK, Simms JA, Li R, Mill D, Yi H, Feduccia AA, Santos N, Bartlett SE (2012). δ-opioid receptor function in the dorsal striatum plays a role in high levels of ethanol consumption in rats. J Neurosci 32(13):4540-52.

Obernier JA, White AM, Swartzwelder HS, Crews FT (2002). Cognitive deficits and CNS damage after a 4-day binge ethanol exposure in rats. Pharmacol Biochem Behav 72, 521–532.

O'Dell LE, Roberts AJ, Smith RT, Koob GF (2004). Enhanced alcohol self-administration after intermittent versus continuous alcohol vapor exposure. Alcohol Clin Exp Res 28(11):1676-82.

Okamoto T, Harnett MT, Morikawa H (2006). Hyperpolarization-activated cation current (Ih) is an ethanol target in midbrain dopamine neurons of mice. J Neurophysiol 95:619-626.

Okun E, Griffioen KJ, Lathia JD, Tang SC, Mattson MP, Arumugam TV (2009). Toll-like receptors in neurodegeneration. Brain Res Rev 59(2):278-92.

Okvist A, Johansson S, Kuzmin A, Bazov I, Merino-Martinez R, Ponomarev I, Mayfield RD, Harris RA, Sheedy D, Garrick T, Harper C, Hurd YL, Terenius L, Ekström TJ, Bakalkin G, Yakovleva T (2007). Neuroadaptations in human chronic alcoholics: dysregulation of the NF-kappaB system. PLoS One 2(9):e930.

Pascual M, Blanco AM, Cauli O, Miñarro J, Guerri C (2007) Intermittent ethanol exposure induces inflammatory brain damage and causes long-term behavioural alterations in adolescent rats. Eur JNeurosci 25(2):541-50.

Pascual M, Baliño P, Alfonso-Loeches S, Aragón CM, Guerri C (2011). Impact of TLR4 on behavioral and cognitive dysfunctions associated with alcohol-induced neuroinflammatory damage Brain Behav Immun 25 Suppl 1:S80-91.

Pascual O, Ben Achour S, Rostaing P, Triller A, Bessis A (2012). Microglia activation triggers astrocyte-mediated modulation of excitatory neurotransmission. Proc Natl Acad Sci 109(4):E197-205.

Pascual M, Baliño P, Aragón CM, Guerri C (2015) Cytokines and chemokines as biomarkers of ethanol-induced neuroinflammation and anxiety-related behavior: role of TLR4 and TLR2. Neuropharmacology 89:352-9.

Paxinos G, Watson C (1998): The rat brain in stereotaxic coordinates, 4th ed. San Diego: Academic Press.

Paxinos G, Watson C (2007) The rat brain in stereotaxic coordinates, 6th ed. Academic Press, Burlington.

Perry VH (1998). A revised view of the central nervous system microenvironment and major histocompatibility complex class II antigen presentation. J. Neuroimmunol 90, 113–121.

Perry VH, Nicoll JA, Holmes C (2010). Microglia in neurodegenerative disease. Nat.Rev.Neurol 6,193–201.

Pfefferbaum, A, Lim, KO, Zipursky, RB, Mathalon, DH, Rosenbloom,MJ, Lane, B, Ha, CN, Sullivan, EV (1992) Brain gray and white matter volume loss accelerates with aging in chronic alcoholics: a quantitative MRI study. Alcohol Clin Exp Res 16, 1078–1089.

Phillips TJ, Brown KJ, Burkhart-Kasch S, Wenger CD, Kelly MA, Rubinstein M, Grandy DK, Low MJ (1998). Alcohol preference and sensitivity are markedly reduced in mice lacking dopamine D2 receptors. Nat Neurosci 1:610-615.

Piao HZ, Choi IY, Park JS, Kim HS, Cheong JH, Son KH, Jeon SJ, Ko KH, Kim WK (2008). Wogonin inhibits microglial cell migration via suppression of nuclear factor-kappa B activity. Int Immunopharmacol 8(12):1658-62.

Plapp BV, Leidal KG, Murch BP, Green DW (2015). Contribution of liver alcohol dehydrogenase to metabolism of alcohols in rats. Chem Biol Interact 234:85-95.

Plapp BV, Leidal KG, Smith RK, Murch BP (1984). Kinetics of inhibition of ethanol metabolism in rats and the rate-limiting role of alcohol dehydrogenase. Arch.Biochem Biophys 230: 30–38.

Plata-Salamán CR, Borkoski JP (1994) Chemokines/intercrines and central regulation of feeding. Am J Physiol 266(5 Pt 2):R1711-5.

Proescholdt MG, Chakravarty S, Foster JA, Foti SB, Briley EM, Herkenham M (2002). Intracerebroventricular but not intravenous interleukin-1beta induces widespread vascular-mediated leukocyte infiltration and immune signal mRNA expression followed by brain-wide glial activation. Neuroscience 112(3):731-49.

Qin L, He J, Hanes RN, Pluzarev O, Hong JS, Crews FT (2008) Increased systemic and brain cytokine production and neuroinflammation by endotoxin following ethanol treatment. J Neuroinflammation 5:10.

Raghavendra V, Tanga FY, DeLeo JA (2004). Attenuation of morphine tolerance, withdrawal-induced hyperalgesia, and associated spinal inflammatory immune responses by propentofylline in rats. Neuropsychopharmacology 29(2):327-34.

Raivich G, Bohatschek M, Kloss CU, Werner A, Jones LL, Kreutzberg GW (1999). Neuroglial activation repertoire in the injured brain: graded response, molecular mechanisms and cues to physiological function. Brain Res. Brain Res Rev 30, 77–105.

Rassnick S, Pulvirenti L, Koob GF (1992). Oral ethanol self-administration in rats is reduced by the administration of dopamine and glutamate receptor antagonists into the nucleus accumbens. Psychopharmacology 109:92-98.

Réaux-Le Goazigo A, Van Steenwinckel J, Rostène W, Mélik Parsadaniantz S (2013) Current status of chemokines in the adult CNS. Prog Neurobiol 104:67-92.

Ren PC, Zhang Y, Zhang XD, An LJ, Lv HG, He J, Gao CJ, Sun XD (2012). High-mobility group box 1 contributes to mechanical allodynia and spinal astrocytic activation in a mouse model of type 2 diabetes. Brain Res Bull 88(4):332-7.

Richardson HN, Zhao Y, Fekete EM, Funk CK, Wirsching P, Janda KD, Zorrilla EP, Koob GF (2008). MPZP: a novel small molecule corticotropin-releasing factor type 1 receptor (CRF1) antagonist. Pharmacol Biochem Behav 88(4):497-510.

Roberto M, Bajo M, Cruz MT, Messing RO, Siggins GR (2008). Ethanol and CRF: which is driving GABA release in the amygdala? Alcoholism and Stress: A Framework for Future Treatment Strategies, Volterra, Italy, May 6-8, 2008.

Roberts AJ, Cole M, Koob GF (1996). Intra-amygdala muscimol decreases operant ethanol self-administration in dependent rats. Alcohol Clin Exp Res 20(7):1289-98.

Rodd-Henricks ZA, McKinzie DL, Crile RS, Murphy JM, McBride WJ (2000). Regional heterogeneity for the intracranial self-administration of ethanol within the ventral tegmental area of female Wistar rats. Psychopharmacology (Berl) 149:217-224.

Rostene W, Kitabgi P, Parsadaniantz SM (2007). Chemokines: a new class of neuromodulator? Nat Rev Neurosci 8:895–903.

Rydberg U (1969) Inhibition of ethanol metabolism in vivo by 4-iodo-pyrazole. Biochem Pharmacol 18(10):2425-8.

Salamone JD, Correa M (2002). Motivational views of reinforcement: implications for understanding the behavioral functions of nucleus accumbens dopamine. Behav Brain Res 137(1–2):3–25.

Schoenbaum G, Shaham Y (2008). The role of orbitofrontal cortex in drug addiction: a review of preclinical studies. Biol Psychiatry 63(3): 256-262.

Schultz W, Dayan P, Montague PR (1997). A neural substrate of prediction and reward. Science 275, 1593–1599.

Schultz W (2007). Behavioral dopamine signals. Trends Neurosci 30:203-210.

Semple BD, Kossmann T, Morganti-Kossmann MC (2010). Role of chemokines in CNS health and pathology: a focus on the CCL2/CCR2 and CXCL8/CXCR2 networks. J Cereb Blood Flow Metab 30(3):459-73.

Simms JA, Steensland P, Medina B, Abernathy KE, Chandler LJ, Wise R, Bartlett SE (2008). Intermittent access to 20% ethanol induces high ethanol consumption in Long-Evans and Wistar rats. Alcohol Clin Exp Res 32(10):1816-23.

Sommer WH, Rimondini R, Hansson AC, Hipskind PA, Gehlert DR, Barr CS, Heilig MA (2008). Upregulation of voluntary alcohol intake, behavioral sensitivity to stress, and amygdala crhr1 expression following a history of dependence. Biol Psychiatry 63(2):139-45.

Sozzani S, Zhou D, Locati M, Rieppi M, Proost P, Magazin M, Vita N, van Damme J, Mantovani A (1994). Receptors and transduction pathways for monocyte chemotactic protein-2 and monocyte chemotactic protein-3. Similarities and differences with MCP-1. J Immunol 152(7):3615-22.

Spanagel R, Rosenwasser AM, Schumann G, Sarkar DK (2005). Alcohol consumption and the body's biological clock. Alcohol Clin Exp Res 29(8):1550-7.

Stamatovic SM, Shakui P, Keep RF, Moore BB, Kunkel SL, Van Rooijen N, Andjelkovic AV (2005) Monocyte chemoattractant protein-1 regulation of blood-brain barrier permeability. J Cereb Blood Flow Metab 25(5):593-606.

Sullivan EV, Marsh L, Mathalon DH, Lim KO, Pfefferbaum A (1995). Anterior hippocampal volume deficits in nonamnesic, aging chronic alcoholics. Alcohol Clin Exp Res 19, 110–122.

Tabakoff B, Saba L, Kechris K, Hu W, Bhave SV, Finn DA, Grahame NJ, Hoffman PL (2008). The genomic determinants of alcohol preference in mice. Mamm Genome 19(5):352-65.

Tanaka T, Minami M, Nakagawa T, Satoh M (2004). Enhanced production of monocyte chemoattractant protein-1 in the dorsal root ganglia in a rat model of neuropathic pain: possible involvement in the development of neuropathic pain. Neurosci Res 48(4):463-9.

Thomas DM, Kuhn DM (2005). Attenuated microglial activation mediates tolerance to the neurotoxic effects of methamphetamine. J Neurochem 92:790–797.

Tieu BC, Lee C, Sun H, Lejeune W, Recinos A 3rd, Ju X, Spratt H, Guo DC, Milewicz D, Tilton RG, Brasier AR (2009) An adventitial IL-6/MCP1 amplification loop accelerates macrophage-mediated vascular inflammation leading to aortic dissection in mice. J Clin Invest 119(12):3637-51.

Tracey KJ (2009). Reflex control of immunity. Nat Rev Immunol 9(6):418-28.

Tynan RJ, Naicker S, Hinwood M, Nalivaiko E, Buller KM, Pow DV, Day TA, Walker FR (2010). Chronic stress alters the density and morphology of microglia in a subset of stress-responsive brain regions. Brain Behav Immun 24(7):1058-68.

Valenta JP, Job MO, Mangieri RA, Schier CJ, Howard EC, Gonzales RA (2013). μ-opioid receptors in the stimulation of mesolimbic dopamine activity by ethanol and morphine in Long-Evans rats: a delayed effect of ethanol. Psychopharmacology (Berl) 228(3):389-400.

Valles SL, Blanco AM, Pascual M, Guerri C (2004) Chronic ethanol treatment enhances inflammatory mediators and cell death in the brain and in astrocytes. Brain Pathol 14(4):365-71.

Vendruscolo LF, Roberts AJ (2014). Operant alcohol self-administration in dependent rats: focus on the vapor model. Alcohol 48(3):277-86.

Vezzani A, Maroso M, Balosso S, Sanchez MA, Bartfai T (2011). IL-1 receptor/Toll-like receptor signaling in infection, inflammation, stress and neurodegeneration couples hyperexcitability and seizures. Brain Behav Immun 25(7):1281-9.

Wakida N, Kiguchi N, Saika F, Nishiue H, Kobayashi Y, Kishioka S (2014) CC-chemokine ligand 2 facilitates conditioned place preference to methamphetamine through the activation of dopamine systems. J Pharmacol Sci 125(1):68-73.

Walker BM, Koob GF (2007) The gamma-aminobutyric acid-B receptor agonist baclofen attenuates responding for ethanol in ethanol-dependent rats. Alcohol Clin Exp Res 31(1):11-8.

Watkins LR, Maier SF (2002). Beyond neurons: evidence that immune and glial cells contribute to pathological pain states. Physiol Rev 82, 981-1011.

Wexler BC, Dolgin AE, Tryczynski EW (1957). Effects of a bacterial polysaccharide (Piromen) on the pituitary-adrenal axis: adrenal ascorbic acid, cholesterol and histologic alterations. Endocrinology 61, 300-308.Zou J, Crews F (2010) Induction of innate immune gene expression cascades in brain slice cultures by ethanol: key role of NF-κB and proinflammatory cytokines. Alcohol Clin Exp Res 34(5):777-89.

Wise RA, Bozarth MA (1987). A psychomotor stimulant theory of addiction. Psychol Rev 94(4):469-92.

Zorrilla EP, Logrip ML, Koob GF (2014). Corticotropin releasing factor: a key role in the neurobiology of addiction. Front Neuroendocrinol 35(2):234-44.

Zou JY, Crews FT (2005). TNF alpha potentiates glutamate neurotoxicity by inhibiting glutamate uptake in organotypic brain slice cultures: neuroprotection by NF kappa B inhibition. Brain Res 1034(1-2):11-24.

Zou J, Crews F (2010). Induction of innate immune gene expression cascades in brain slice cultures by ethanol: key role of NF-κB and proinflammatory cytokines. Alcohol Clin Exp Res 34(5):777-89.

Zou J, Crews FT (2012) Inflammasome-IL-1β Signaling Mediates Ethanol Inhibition of Hippocampal Neurogenesis. Front Neurosci 30; 6:77.

Zou J, Vetreno RP, Crews FT (2012). ATP-P2X7 receptor signaling controls basal and TNFalpha-stimulated glial cell proliferation. Glia 60(4): 661-673.

Vita

John Valenta's mother and father immigrated from Poland and Czech Republic, respectively. John and his brother are the first in their lineage to attend college, both earning doctorate degrees.

John's key achievements during his doctorate program include publishing two first author manuscripts; winning the Gordis Research Recognition Award from the Research Society on Alcoholism; winning Best Poster, Honorable Mention at the Gordon Research Conference; receiving a score of "10" on his National Research Service Award application; and being elected President of the Psychology Graduate Student Association.

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